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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 33/567 — A1

(11) International Publication Number: WO 00/62067

(43) International Publication Date: 19 October 2000 (19.10.00)

(21) International Application Number: PCT/US00/05097

(22) International Filing Date: 28 February 2000 (28.02.00)

(30) Priority Data:

60/122,757	28 February 1999 (28.02.99)	US
60/151,291	29 August 1999 (29.08.99)	US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NOVEL TRANSDUCTION MOLECULES AND METHODS FOR USING SAME

(57) Abstract

The invention relates to novel fusion molecules and methods for introducing the fusion molecules into a desired cell, tissue or organ.

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NOVEL TRANSDUCTION MOLECULES AND METHODS FOR USING SAME

CROSS-REFERENCE TO RELATED APPLICATION

The present application is a continuation-in-part of co-pending U.S. Provisional Application Serial No. 60,122,757 filed on February 28, 1999 and U. S. Provisional Application Serial No. 60/151,291 filed on August 29, 1999 both of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to novel transduction molecules as well as methods for introducing same into a desired cell, tissue, or organ of a mammal including providing for introduction of the molecules into essentially the entire mammal. The present invention has a wide variety of useful applications including providing targeted and highly efficient entry into cells of a wide spectrum of therapeutic molecules such as vaccines, anti-infectious drugs, anti-tumor drugs, and agents having pharmacological activity in the nervous system.

The invention more particularly provides transduction molecules for transducing very large molecules into a mammalian cell, tissue or organ up to transduction of essentially an entire living mammal. In one embodiment, what is sometimes referred to herein as a *"Blue"* mouse is a particularly useful test system for detecting and characterizing transduction of at least one molecule of interest in the whole animal. Additionally provided are novel molecule discovery strategies that utilize the compositions and methods provided herein. Preferred use of the discovery strategies can detect new therapuetic molecules including those believed to be missed by prior drug screens.

Also provided are methods for renewed use of recognized therapuetic molecules that have succumbed to emergence of resistant

strains. In one embodiment, the methods can be used to transduce an antibiotic through cell membranes, thereby helping to evade resistance mechanisms and restoring therapuetic value to the antibiotic.

2. Background

There have been attempts to develop methods for introducing heterologous molecules into cells. For example, a majority of the methods focus on introducing a nucleic acid and then expressing that nucleic acid inside cells to generate heterologous protein. See generally, Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed.); and Ausubel et al., *Current Protocols in Molecular Biology*, (1989) John Wiley & Sons, New York.

However, the prior methods are generally not useful for introducing large nucleic acid and non-nucleic acid molecules into cells. For example, use of the methods to introduce molecules larger than about 600 to 700 Da ("large molecules") typically has been unsuccessful and often fatal to the cells. Difficulties are magnified when substantial amounts of a large molecule must be introduced into the cells.

There is recognition that the efficiency by which many molecules can be introduced into cells is constrained by parameters such as bioavailability. In particular, bioavailability is understood to be a summation of characteristics such as molecular weight, hydrophilicity, hydrophobicity, charge, and biological resistance.
For example, attempts to introduce pharmacologically relevant peptidyl mimetics and/or proteins and polypeptides have been hampered by size constraints that limit bioavailability when in excess of about 0.5 to 1 kDa. Also, molecules having a size less than about 400 to 600 Da ("small molecule") and significant hydrophobicity will have limited bioavailability in many instances.

There has been particular recognition that biological resistance can pose a formidable threat to bioavailablility. See generally Gilman et

al. (1993) in *The Pharmacological Basis of Therapeutics*, (8th Ed.) and references cited therein.

As an illustration, the central nervous system (CNS) is known to defend itself against efficient entry of many molecules (large and small) by a structure called the "blood-brain barrier". More specifically, efficient introduction of many therapeutic molecules into the brain is often resisted by the blood-brain barrier.

There have been attempts to overcome the resistance posed by the blood-brain barrier. For example, one approach has been to permeabilize the barrier or using high amounts of the molecule of interest. However, practice of these methods has been associated with potentially life threatening side effects such as seizures and CNS inflammation. See Gilman, et al. (1993), *supra*, pages 1-62.

There have been reports that biotransformation and immune surveillance facilitate biological resistance and can negatively impact the bioavailability of more particular therapeutic molecules such as drugs, vaccines, peptidyl mimetics and/or therapeutic proteins and polypeptides.

There have been some attempts to develop alternative methods for introducing large molecules into target cells, tissues and organs. One approach involves genetically fusing amino acid sequences to a specified transfer molecule and then introducing the resulting construct fusion into cells. For example, See e.g., Frankel, A.D. and C.O. Pabo (1988) *Cell* 55:1189; Fawell, S. et al. (1994) *PNAS (USA)* 91: 664; Chen, L.L. et al. *Anal. Biochem.*, 227:168 (1995); U.S. Pat. No. 5,652,122.; and PCT WO/04686.

However, the prior methods have been associated with significant problems that have negatively impacted widespread use of the methods.

For example, many of the prior methods are not capable of transducing sufficient amounts of fusion proteins into target cells. Intracellular amounts of the fusion protein and their biological activities can therefore be limited. Attempts to overcome these and related problems have included transducing large amounts of fusion proteins into target cells. However, that strategy can sometimes be lethal to transduced cells. In addition, rates of transduction exhibited by prior transduction methods often have been less than optimal.

Biological resistance has especially impacted the bioavailability of anti-microbial agents. For example, there is nearly universal agreement that microbes have become very adept at developing resistance to a wide array of antibiotics. Significantly resistance to multiple antibiotics has been reported to be infectious, thereby posing a significant public health emergency in the United States and particularly abroad.

There have been attempts to decrease or eliminate biological resistance to anti-microbial agents. For example, one approach calls for manipulating the agents themselves usually by chemical modification to make subsequent compounds, ie., so called second or third generation drugs. However, this approach has been costly and has not always been sufficient to curtail resistance. Sometimes this approach has encouraged emergence of so-called "super bugs", some of which have been reported to be almost completely resistant to most standard anti-microbial therapies.

Accordingly, it would be useful to have methods for introducing a fusion molecule into a mammal that can be targeted to a desired cell, tissue, or organ up essentially all cells in the mammal. It would be especially desirable to have methods for introducing a broad spectrum of molecules into the mammal, preferably at targeted sites, such as therapeutic molecules having unknown or poor bioavailability. Further desirable would be to have animal models for testing or validating

therapeutic agents useful in the diagnosis or treatment of medical condition.

SUMMARY OF THE INVENTION

The present invention relates to novel fusion molecules and methods for introducing the molecules into a desired cell, tissue, or organ including introducing same into essentially all of the cells of a mammal. In one aspect, the invention features "macro-transduction" methods that include transducing the fusion molecule into the organ or group of organs in the mammal up to essentially the entire mammal. In another aspect, the invention provides "micro-transduction" methods including transducing the fusion molecule into one or a few cells or tissue in the mammal. The present invention has several useful applications including providing animal models for testing therapeutic agents useful in the diagnosis or treatment of a medical condition.

In one aspect, the invention provides novel fusion molecules that include nearly any molecule that can be covalently linked (ie.fused) to a protein or peptide. The molecule can be naturally-occurring or it can be made by synthetic or semi-synthetic routes. Illustrative fusion molecules are disclosed throughout the specification including the discussion, examples and drawings that follow. For example, in one embodiment, the fusion molecule can be a fusion protein as provided below. Also, the fusion molecule can include at least one of a vaccine, steroid or peptide hormone, anesthetic, analgesic, anti-inflammatory, anti-diabetic, anti-infectious agent, anti-tumor drug, cardiovascular drug, vitamin, drug with pharmacological activity in the nervous system especially the peripheral nervous system (PNS) and central nervous system (CNS), or a diagnostic marker.

More particular fusion molecules in accord with this invention include fused drugs with recognized treatment or prophylactic activity against a recognized medical condition in a human patient. Exemplary of such drugs include those with suspected or known activity in the

treatment or prevention of infection (antibiotics), the treatment of cancer (chemotherapuetic drugs), pain management (e.g., analgesics and opiates), cardiovascular drugs, and the treatment of gastrointestinal ailments.

Additional drugs of interest are those which have or are suspected of having poor bioavailability including those which exhibit unacceptable lipid solubility, biotransformation including plasma clearance, hydrophilicity, size, or which are subject to attack by the immune system.

Of additional particular interest are those fusion molecules including at least one fused drug which has or is suspected of having significant therapeutic activity in the CNS and especially the brain. Specifically contemplated are those drugs which do not demonstrate appropriate bioavailability. Particularly contemplated are those drugs which feature a recognized inability to cross the blood brain barrier in a subject mammal such as a human. Of more particular interest are those drugs which have or are suspected of having capacity to treat or alleviate symptoms associated with nervous system dysfunction, e.g., Alzheimer's disease, pre-senile dementia including age-related memory loss, Huntington's disease, Parkinson's disease, meningitis including bacterial meningitis, and encephalitis. Additionally contemplated are those drugs which have or are suspected of having therapeutic activity in the management of Lou Gehring's disease and related conditions.

Additional drugs of interest for making the fusion molecules include those which when administered as described herein can complement an existing physiological defect especially in the nervous system and particularly the brain. Examples include those fusion molecules and especially fusion protein which can provide a specified protein which is not present in suitable quantities in the targeted cell, tissue, organ or entire mammal.

Additional fusion molecules of this invention can be adapted to provide still further therapeutic benefits such as facilitating visualization of a specific cell, tissue or organ in the mammal. Examples include those molecules that include a detectably-labeled moiety such as a fluorescent, chemiluminescent, chromogenic or radioactive tag.

As will be discussed below, preferred fusion molecules of this invention include at least one synthetic or semi-synthetic protein transduction domain (PTD) sometimes referred to herein as SFD or STD) linked to the protein, peptide, drug, or other linked molecule of interest as provided herein. In some instances such as those in which spacing between the PTD and linked molecule is desirable, the PTD is fused to a suitable linker sequence which sequence is fused to the linked molecule. Illustrative linkers include amino acid sequences discussed below.

As also will be discussed below, preferred fusion molecules of this invention include at least one site which is cleavable by a pre-determined cell or group of cells up to essentially the entire mammal as needed so that the linked molecule can be freed from the PTD. However for some applications such as those involving linkage of a diagnostic agent addition of that cleavable site may not be necessary. More specific disclosure relating to release of several linked molecules is provided below.

Preferred fusion molecules can be administered to a subject mammal and especially a primate such as a human patient using nearly any acceptable route including the "macro-transduction" or "micro-transduction" approaches disclosed herein. Methods for making the fusion molecules involve standard manipulations discussed in more detail below.

In addition, the present invention relates to highly efficient methods for introducing a desired fusion molecule into a cell, group of

cells including tissue, organ, group of organs, physiological system up to essentially an entire mammal. That is, the invention encompasses methods that are highly flexible and can be conveniently tailored to fit the specificity of transduction desired by varying dose of the fusion molecule. For example, disclosed are "macro-transduction "methods that can be used to transduce a desired fusion molecule into desired organ using a high dose of the fusion molecule. Quite suprisingly, practice of the disclosed methods can provide for efficient transduction of the fusion molecule into essentially the entire mammal as required.

Also provided are what is referred to herein as "micro-transduction" methods. More particularly, is possible to introduce a desired fusion molecule into at least one cell in the mammal up to a few cells including tissue by administering the fusion molecule in a small dose. As will be discussed below, practice of the methods can form a damage or killing zone around the cells or tissue of interest. Preferred use of the methods encompasses what is sometimes referred to herein as "molecular surgery". As will be discussed below, this technique is especially useful for reducing or eliminating undesired cell densities that do not rely on performing highly invasive and painful surgical manipulations.

Accordingly, the fusion molecules and methods disclosed herein solve a long felt need in the field for compositions and methods for using same that can be used to introduce a desired fusion molecule into just one or a few cells and tissue or essentially all of cells of the mammal.

Further provided by this invention are mammals that in one embodiment can be used to test and optionally validate therapeutic agents useful in the diagnosis or treatment of a medical or veterinary condition. A preferred mammal is typically a primate, rodent, rabbit or other suitable animal model into which has been introduced at least one and usually one fusion molecule of this invention.

Particular mammals of interest include those having at least one fusion molecule transduced into a cell, group of cells including tissue, organ, group of organs, a physiological system up to essentially the entire mammal. In many embodiments, the cell, tissue, organ or system will be pre-determined. Illustrative systems are known in the field and include the circulatory system particularly including the heart and major blood vessels, lymphatic system, immune system, pulmonary system, and the nervous system and especially the PNS and/or CNS (particularly the brain and spinal cord). Methods for determining efficient transduction are described below and include recognized immunological and immunohistochemical techniques.

In another aspect, the invention provides specific kits that include at least one of the mammals described above along with at least one of the fusion molecules disclosed herein. In one embodiment, the kit also includes one or more implementations for introducing the fusion molecule into the mammal such as a syringe and directions for using the kit. Additionally provided in some instances are vectors encoding all or part of the fusion molecule. Preferred kits provide the fusion molecules as sterile formulations acceptable for mammalian use. A preferred mammal is a rodent and particularly a mouse, rat, or a rabbit.

The invention further provides methods for treating or preventing a medical condition in a mammal which includes administering to the mammal a therapeutically effective amount of at least one of the fusion molecules described herein including specific fusion proteins, the amount being sufficient to treat or prevent the condition in the mammal.

Additionally provided are methods for testing at least one and preferably one fusion molecule for therapeutic capacity to treat or prevent a medical condition in a mammal, the method including administering at least one of the fusion molecules disclosed herein an amount sufficient to treat or prevent the medical condition.

Mammals especially suited for use in the methods include recognized test mammal such a certain primates, rodents and rabbits. More specific examples include mice and rats. Preferred medical conditions include an infection particularly facilitated by a microbe or virus, neurological disorder such as those afflicting the PNS or CNS. Especially preferred medical conditions include those for which a recognized cell or animal model for studying the condition is available.

Also provided are methods for treating or preventing infection by an infectious agent which methods include administering to the mammal a therapeutically effective amount of at least one of the fusion molecules discussed herein. Particular infectious agents are described below and include infection by one or more of a pathogenic virus, yeast, bacterium, nematode, fungus, helminth, prion, or protozoan.

The present invention provides several significant advantages. For example, the invention provides, for the first time, a highly effective means of targeting protein transduction to essentially an entire mammal or to smaller parts thereof such as a cell, tissue, organ, or group of organs in that mammal. This feature of the invention is a substantial advance of prior methods which were do not always result in efficient transduction.

Significantly, the present invention reduces or essentially eliminates many known or suspected bioavailability problems that plague effective utilization of recognized therapeutic agents as well as those being developed. In particular, this invention removes problems stemming from unacceptable bioavailability, e.g., large molecular size, hydrophobicity, hydrophilicity, and/or biological resistance; by providing for highly efficient transduction of the fusion molecules into target cells. Significantly, the methods of this invention are not substantially impacted by the prior bioavailability problems.

As one illustration of these and other advantages, the invention provides for transduction of large molecules some of which may also feature unacceptable water or lipid solubility. Heretofore, there has not been a widely practiced method for using these molecules in many clinical settings. This invention positively impacts utilization of these molecules by providing for highly efficient and target transduction into desired cells, tissues, organs, systems, up to essentially the entire mammal. In particular, practice of this invention will help most current drug discovery efforts by providing methods of using drugs with known or suspected bioavailability problems. In accord with this invention, many of these molecules can now be efficiently transduced into a subject mammal in an essentially global (macro-transduction) or highly targeted (micro-transduction) manner. Accordingly, the present invention substantially expands the existing medical armamentarium with new fusion molecules comprising the drugs. Also, practice of this invention can help to reduce resources needed to bring a new therapeutic drugs into compliance with federal regulations and into clinical use.

As discussed, the present invention provides novel fusion molecules and methods for using same that reduce or eliminate biological resistance to a variety of therapeutic drugs. For example, there is provided methods for significantly increasing transduction of molecules having poor bioavailability in the CNS and particularly the brain. In this instance, problems relating to drug passage through the blood brain barrier are significantly reduced or totally eliminated by the novel fusion molecules and highly efficient transduction methods provided by this invention. Thus, the invention facilitates implementation of a variety of therapeutic strategies in the brain and elsewhere in the mammal which until now have been difficult or impossible to practice.

As also discussed, the invention provides highly effective methods for transducing at wide variety of compounds into a pre-determined site in the subject mammal including essentially the entire mammal if

needed. Significantly, the novel fusion molecules and methods of this invention can reduce or totally eliminate microbial resistance to many of recognized anti-infective agents and particularly antibiotics. For example, the invention provides methods for using previously discarded or underutilized antibiotics such as the penicillins, tetracyclines, sulfa drugs ect., which can be resisted by many targeted microbes. Emergence of powerful microbial defenses against these and other anti-infective agents have helped to accelerate the disuse. Practice of this invention significantly evades these and related defenses by providing efficient transduction of the fusion molecules into the microbe and particularly into the highly sensitive cell cytoplasm.

In particular, the fusion molecules and methods for using same provided by this invention allow for more potent contact with the cytoplasm of many microbes. That cytoplasm is known to house a wide spectrum of sensitive targets such as organelles and particularly, ribosomes, nucleus, Golgi, mitochondria, ect., as well as key synthetic enzymes such as those involved with DNA, RNA and protein synthesis. The invention thus provides methods for efficiently contacting these targets, thereby substantially boosting effectiveness of the fusion molecules. Microbes which attempt to evade therapeutic intervention by formation of resistant capsules such as spores, cysts, ect. can also be treated or prevented by use of this invention.

More generally, in embodiments in which the fusion molecule includes at least one cleavable site for releasing the linked molecule, that site can be designed for cleavage inside target cells. In this example, prior bioavailability problems relating to immune surveillance and biotransformation (e.g., plasma clearance) can be substantially reduced or eliminated because the linked molecule (drug) is efficiently delivered inside desired cells. Accordingly, for many fusion molecules, optimal dosages for therapy can be reduced resulting in lowered side-effects and better patient tolerance.

In another aspect, the present invention relates to an anti-pathogen system that exhibits high transduction efficiency and specifically kills or injures cells infected by one or more pathogens. In general, the anti-pathogen system includes a fusion molecule that comprises a transduction domain and a cytotoxic domain genetically and hence covalently linked together as an in-frame fusion molecule. The invention further relates to transduction domains that enhance the transduction efficiency of the fusion molecules. The anti-pathogen system is essentially inactive in uninfected cells but it is specifically activated in cells infected by the pathogen. Further provided are methods of using the anti-pathogen system to treat infection by a pathogen and particularly human pathogens such as certain viruses and plasmodia.

Preferred use of the anti-pathogen system entails that the pathogen infection induce at least one pathogen specific protease. Preferably, that protease is capable of specifically cleaving a target amino acid sequence. The target amino acid sequence is one component of the fusion molecule and it is sometimes referred to herein as a protease recognition or cleavage site. Specific cleavage of the protease recognition site cleaves the fusion molecule, generally at or near the cytotoxin domain, to form a cytotoxin. The cytotoxin so formed is specifically capable of killing or injuring cells infected by the pathogen.

Significantly, the present anti-pathogen system links formation of the cytotoxin to presence of the pathogen-induced protease, thereby providing highly focussed cytotoxic action to infected cells. Formation of the cytotoxin is minimized or eliminated in uninfected cells and in infected cells that keep the pathogen inactive. The anti-pathogen system is therefore capable of effectively and specifically discriminating between productively infected and uninfected cells.

The present anti-pathogen system has a number of important advantages. For example, it can be readily manipulated to respond to

changes in pathogen serotype. That is, the anti-pathogen system can be specifically tailored to kill or injure cells infected by one or more pathogen strains. In contrast, prior methods of blocking infection and especially drug-based methods are not usually designed to respond to changes in pathogen serotype. This deficiency often results in uncontrolled growth of drug-resistant pathogen strains. As will become more apparent from the discussion that follows, the anti-pathogen system has capacity to harness production of one or more pathogen-induced protease to kill or injure cells infected by the pathogen serotype. In marked contrast, most prior drug-based methods merely attempt to inhibit pathogenic processes, e.g., by blocking activity of a pathogen gene product. The present anti-pathogen system is more flexible and can be used to reduce or even eliminate emergence of pathogen strains by specifically exposing infected cells to cytotoxin.

As an illustrative example of the flexibility of the present invention, the anti-pathogen system is particularly useful against emergence of HIV serotypes. For example, many patients infected by HIV manifest several viral strains. Conventional drug-based therapies usually attempt to block activity of an HIV enzyme such as RT or an HIV protease. The clinical outcome of such treatment is often emergence of a spectrum of HIV serotypes. It has been recognized that the HIV serotypes can develop partial or even complete resistance to the therapies. Even so-called "cocktail" therapies employing multiple anti-HIV drugs have been problematic. In contrast, the anti-pathogen system of the present invention is highly flexible and can be adapted to kill or injure cells that produce the HIV serotypes by employing HIV proteases. Significantly, the anti-pathogen system is also formatted to meet an increase in the activity of those HIV proteases or an increase in the number of infected cells with enhanced activation of the system.

The flexibility of the present anti-pathogen system arises in part because it can be tailored to kill or injure cells infected by nearly any number of HIV serotypes. Thus it is possible in accord with the present

invention to format the anti-pathogen system to combat one or more HIV strains in a particular patient. This feature is highly useful in several respects. For example, it provides a specific method of fighting an HIV infection in a single patient without resorting to administration of potentially harmful or ineffective drugs. Significantly, the anti-pathogen system can be formatted to be effective at nanomoler doses or less. This low level of anti-viral activity is significantly lower than many present drug-based therapies. This feature of the invention positively impacts patient tolerance for the anti-pathogen system.

Further, the present anti-pathogen system is fully compatible with recognized anti-HIV therapies such as those using a "cocktail" format (ie. combination of anti-HIV drugs) to kill or injure infected cells.

In particular embodiments of the present invention, the anti-pathogen system is employed to reduce or eliminate emergence of HIV serotypes by exploiting the HIV protease produced by the virus.

Illustrative fusion proteins that kill HIV-infected cells are provided in Examples 11 and 12 below.

In addition, the present anti-pathogen system is capable of transducing unexpectedly large fusion molecules into cells. In particular, it has been discovered that the anti-pathogen system accommodates misfolded (i.e. partially or completely unfolded) fusion molecules and provides for efficient transduction of those molecules into cells. In particular, it is believed that the anti-pathogen system is compatible with misfolded fusion molecules having a molecular weight in the range of about 1 to about 500 kDa or more. The anti-pathogen system therefore is widely applicable to transducing a large spectrum of fusion molecules into cells.

More specifically, the ability to transduce misfolded fusion molecules has substantial advantages over prior transduction methods.

For example, it has been found that misfolded fusion proteins used in accord with this invention significantly enhance transduction efficiency sometimes by as much as about 10 fold or greater. In addition, by misfolding the fusion proteins, it has been found that it is possible to optimize the amount of the fusion molecules inside cells. Preparation and storage of the fusion molecules are also positively impacted by the misfolding.

As discussed, the present anti-pathogen system is flexible. For example, it is not limited to any particular type of pathogen or cell provided that the pathogen is capable of inducing at least one specified protease in that cell. The protease can be a pathogen-induced or host cell induced protease that is specifically induced (i.e. synthesized or activated) in response to the infection. However, the specified protease must be capable of cleaving the protease recognition site on the fusion molecule to activate the cytotoxin.

The present anti-pathogen system and methods of using same can be used *in vitro* or *in vivo*. Further, the order or number of components of the fusion molecule are not important so long as each component on the molecule is operatively linked and can perform specified functions for which it is intended.

The cytotoxin produced by the anti-pathogen system is preferably selected to kill or injure infected cells in the presence of one or more of cell proteases and usually the pathogen- or host cell induced proteases. Preferably, the cytotoxin can kill at least about 20%, 25%, 50%, 75%, 80%, or 90% of the cells and preferably up to about 95%, 98% or 100% of the cells infected by the pathogen as assayed by standard cell viability tests. A preferred viability test is a standard Trypan Blue exclusion assay although other assays may be used as needed. It is also preferred that the cytotoxin activity be limited to cells in which it is produced.

As noted previously, the present anti-pathogen system includes an in-frame fusion molecule. The fusion can be accomplished by conventional recombinant nucleic acid methods. If desired, the fusion can also be achieved by chemically linking the transducing protein to the cytotoxic domain according to conventional methods described below.

In general, the transduction domain of the fusion molecule can be nearly any synthetic or naturally-occurring amino acid sequence that can transduce or assist in the transduction of the fusion molecule. For example, transduction can be achieved in accord with the invention by use of a protein sequence and particularly an HIV TAT protein or fragment thereof that is covalently linked to the fusion molecule. Alternatively, the transducing protein can be the *Antennapedia* homeodomain or the HSV VP22 sequence, or suitable transducing fragments thereof such as those known in the field.

The type and size of the transducing amino acid sequence will be guided by several parameters including the extent of transduction desired. Preferred sequences will be capable of transducing at least about 20%, 25%, 50%, 75%, 80% or 90% of the cells of interest, more preferably at least about 95%, 98%% and up to about 100% of the cells. Transduction efficiency, typically expressed as the percentage of transduced cells, can be determined by several conventional methods such as those specific microscopical methods discussed below (e.g., flow cytometric analysis).

Additionally preferred transducing sequences will manifest cell entry and exit rates (sometimes referred to as k_1 and k_2, respectively) that favor at least picomolar amounts of the fusion molecule in the cell. The entry and exit rates of the amino acid sequence can be readily determined or at least approximated by standard kinetic analysis using detectably-labeled fusion molecules. Typically, the ratio of the entry rate to the exit rate will be in the range of from between about 5 to about 100 up to about 1000.

Particularly are transducing amino acid sequences that include at least a peptide featuring substantial alpha-helicity. It has been discovered that transduction is optimized when the transducing amino acid sequence exhibits significant alpha-helicity. Also preferred are those sequences having basic amino acid residues that are substantially aligned along at least one face of the peptide. Typically such preferred transduction sequences are synthetic protein or peptide sequences.

More preferred transducing amino acid sequences are referred to as class I transducing domains or like term and include a strong alpha helical structure with a trace of arginine (Arg) residues down the helical cylinder.

In one embodiment, the class I transducing domain is a peptide is represented by the following general formula: B1 -X_1-X_2-X_3-B_2-X_4-X_5-B_3; wherein B_1, B_2, and B_3 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3, X_4 and X_5 are each independently an alpha-helix enhancing amino acid the same or different.

In another embodiment, the class I transducing peptide is represented by the following general formula: B_1 -X_1-X_2-B_2 -B_3 -X_3-X_4-B_4; wherein B_1, B_2, B_3, and B_4 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3, and X_4 are each independently an alpha-helix enhancing amino acid the same or different.

Additionally preferred transducing peptides are often referred to herein as "class II" domains or like terms. These domains generally require basic residues, e.g., lysine (Lys) or arginine (Arg), preferably arginine (Arg), and further including at least one proline (Pro) residue sufficient to introduce "kinks" into the domain.

In one embodiment, the class II domain is a peptide represented by the following sequence: X-X-R-X-(P/X)-(B/X)-B-(P/X)-X-B-(B/X), wherein X is any alpha helical promoting residue, preferably alanine; P/X is either proline or X as previously defined; B is a basic amino acid residue, e.g., arginine (Arg) or lysine (Lys), preferably arginine (Arg); R is arginine (Arg) and B/X is either B or X as defined above.

Although for many applications at least one PTD and usually one PTD will be preferred to make the fusion molecules disclosed herein, the invention also provides for use of a transducing TAT fragment. Particularly preferred are the synthetic PTDs disclosed throughout this application.

For example, additional transducing sequences in accord with this invention include a TAT fragment that comprises at least amino acids 49 to 56 of TAT up to about the full-length TAT sequence. A preferred TAT fragment includes one or more amino acid changes sufficient to increase the alpha-helicity of that fragment. In most instances, the amino acid changes introduced will involve adding a recognized alpha-helix enhancing amino acid. Alternatively, the amino acid changes will involve removing one or more amino acids from the TAT fragment the impede alpha helix formation or stability. In more specific embodiment, the TAT fragment will include at least one amino acid substitution with an alpha-helix enhancing amino acid. Preferably the TAT fragment will be made by standard peptide synthesis techniques although recombinant DNA approaches may be preferred in some cases.

Additional transduction proteins of this invention include the TAT fragment in which the TAT 49-56 sequence has been modified so that at least two basic amino acids in the sequence are substantially aligned along at least one face of the TAT fragment and preferably the TAT 49-56 sequence. In one embodiment, that alignment is achieved by making at least one specified amino acid addition or substitution to the TAT 49-56 sequence. Illustrative TAT fragments include at least one specified amino

acid substitution in at least amino acids 49-56 of TAT which substitution aligns the basic amino acid residues of the 49-56 sequence along at least one face of the segment and preferably the TAT 49-56 sequence.

Additional transduction proteins in accord with this invention include the TAT fragment in which the TAT 49-56 sequence includes at least one substitution with an alpha-helix enhancing amino acid. In one embodiment, the substitution is selected so that at least two basic amino acid residues in the TAT fragment are substantially aligned along at least one face of that TAT fragment. In a more specific embodiment, the subitution is chosen so that at least two basic amino acid residues in the TAT 49-56 sequence are substantially aligned along at least one face of that sequence.

Additionally provided are chimeric transducing proteins that include parts of at least two different transducing proteins. For example, chimeric transducing proteins can be formed by fusing two different TAT fragments, e.g., one from HIV-1 and the other from HIV-2. Alternatively, other transducing proteins can be formed by fusing a desired transducing protein to heterologous amino acid sequences such as 6XHis, (sometimes referred to as "HIS"), EE, HA or Myc.

As noted above, the fusion molecules of the present invention can also include a fused cytotoxic domain. In general, the cytotoxic domain includes a potentially toxic molecule and one or more specified protease cleavage sites. By the term "potentially toxic" is meant that the molecule is not significantly cytotoxic to infected or non-infected cells (preferably less than about 30%, 20%, 10%, 5%, 3%, or 2% cell mortality as assayed by standard cell viability tests. More preferred is 1% or less cell mortality) when present as part of the cytotoxic domain. As noted above, the protease cleavage sites are capable of being specifically cleaved by one or more than one of the proteases induced by the pathogen infection.

In some embodiments of this invention, it will be useful if the protease cleavage sites are selected to remain essentially uncleaved in uninfected cells, thereby maintaining the cytotoxic domain in an inactive state. These protease cleavage sites may also be selected to remain essentially uncleaved in cells in which the pathogen is inactive. However, in the presence of a specified pathogen-induced or host cell induced protease, the protease cleavage sites are specifically cleaved to produce a cytotoxin from the potentially toxic molecule. That is, cleavage of the protease sites releases the cytotoxic domain from the fusion molecule, thereby forming an active cytotoxin. The one or more protease cleavage sites are generally positioned in the cytotoxic domain to optimize release of all or part of the domain from the fusion protein and to enhance formation of the cytotoxin.

More preferred protease cleavage sites for use in this embodiment are selected so as not to be cleaved by a protease normally associated with an uninfected cell. These proteases have been generically referred to as "housekeeping" proteases and are well known.

Protease cleavage sites are sometimes referred to herein as "pathogen-specific" cleavage sites to denote capacity to be specifically cleaved by one or more proteases induced by the pathogen infection. The protease cleavage sites are "responsive" to a pathogen (or more than one pathogen) insofar as cleavage of those sites releases the cytotoxin domain from the fusion molecule, thereby activating the cytotoxin.

As discussed however, in other embodiments of this invention it will be useful to construct fusion molecules which include one or more cleavage sites for housekeeping or related proteases found inside one cell or group of cells up to all the cells of the subject mammal. In this example, the molecule linked to the fusion molecule can be at least one and usually one protein, polypeptides, drug, or other agent which molecule is released once inside target cells. In preferred examples, the molecule is then provided to the cells and can remain prophylactically if

needed, e.g., to kill or injure a targeted cell, tissue or organ; or to complement a known or suspected deficiency inside the cell such lack or insufficient amount of a needed nucleic acid, protein, or lipid. Methods for making and using such fusion molecules are provided below.

In embodiments in which the fusion molecule includes a cytotoxic domain, that domain can include one or more of a variety of potentially toxic molecules provided that it can be released from the fusion molecule as discussed. An illustrative cytotoxic domain for use in the fusion molecules includes an immature enzyme. These immature enzyme is sometimes referred to as zymogen, proenzyme, preproenzyme or simply as "pre-" "pre-pro" or "pro-" forms of more mature enzyme. Preferred zymogens can be specifically activated to a cytotoxin (ie. a cytotoxic enzyme) by site-specific proteolysis at one or more naturally-occuring protease cleavage sites on the zymogen. The zymogens can be further processed in some instances by self-proteolysis.

Particularly, a cytotoxic domain that includes a preferred zymogen will include one or more specified protease cleavage sites that have been added within and/or around the zymogen. The cleavage sites are optionally positioned to facilitate release and processing of the zymogen to a mature or more mature cytotoxic enzyme.

In this particular example, the addition of certain protease cleavage sites to the zymogens can be supplative with respect to the naturally-occurring protease cleavage sites in that zymogen. However it is preferred that the cleavage sites be substituted for one or more of the naturally-occurring cleavage sites. In this embodiment, the substituted protease cleavage sites in the zymogen are capable of being specifically cleaved by one or more pathogen-specific proteases. It has been found that by partially or completely substituting the naturally-occurring protease cleavage sites of the zymogen with one or more pathogen responsive cleavage sites, maturation of the zymogen into a cytotoxin is brought under substantial or complete control by the pathogen infection.

A variety of specific zymogens are suitable for inclusion in the cytotoxic domain as discussed below. Active forms of those zymogens generally include bacterial toxins and particularly exotoxins, plant toxins, and invertebrate toxins including conotoxins, snake and spider toxins.

Further contemplated cytotoxic domains include known proteins with potential to exert genetically dominant characteristics. That is, the proteins can be specifically cleaved from the fusion protein and can subsequently override one or more cell functions such as cell replication. In this embodiment, the potentially dominant protein must not manifest the dominant characteristic (sometimes known as a dominant phenotype) until that protein is released from the fusion protein. Examples of potentially dominant proteins in accord with the invention include proteins that inhibit cell replication such as the retinoblastoma protein (Rb), p16 and p53.

Further contemplated cytotoxic domains include essentially inactive enzymes that have capacity to convert certain nucleosides or analogs thereof into a cytotoxin. In this embodiment, the cytotoxic domain will include one or more specified protease cleavage sites, that is preferably positioned to release the inactive enzyme from the fusion protein. Following the release, the enzyme converts the nucleoside or analog thereof into a cytotoxin. Examples of such enzymes include viral thymidine kinase and nucleoside deaminases such as cytosine deaminase. Also contemplated are cytotoxic domains comprising catalytically active fragments of the enzymes such as those generally known in the field.

The present anti-pathogen system provides a number of additional important advantages. For example, the anti-pathogen system unexpectedly accommodates misfolded fusion proteins. As will become more apparent from the discussion and examples which follow, that

feature has been found to substantially boost levels of the fusion protein inside cells. Typically, a corresponding increase in the amount of administered fusion protein is not required. Without wishing to be bound to theory, it is believed that transduction of misfolded fusion molecules and especially provided fusion proteins requires modest numbers of molecules and only a few of those need be refolded to manifest an effective cytotoxic effect. For example, it is believed that with certain preferred fusion proteins such as those described below in Examples 5-6, only about 10 to 100 correctly refolded fusion proteins are needed to kill or injure infected cells. Thus, the present invention can decrease or even eliminate the need to concentrate large number of cytotoxic molecules inside cells to achieve significant anti-pathogen activity.

In addition, it has been found that activity of the present anti-pathogen system as well as the novel fusion molecules disclosed herein are enhanced by mass action.

More particularly for certain fusion molecules, it has been found that specific cleavage of the cytotoxic domain can draw additional fusion molecules into infected cells. This feature can be particularly advantageous for those fusion proteins that include cytotoxic domains which are preferably administered in sub-optimal doses. In such instances, the fusion protein is specifically concentrated in infected cells, thereby increasing levels of the cytotoxin to lethal or near lethal levels. Importantly, the cytotoxin remains at sub-optimal levels in uninfected cells.

Still further advantages are provided with respect to particular fusion proteins of the invention that include the TAT fragment described above. For example, the cytotoxic domain of a protein fused to the TAT fragment need not be directed to the cell nucleus or to RNA. More specifically, the present fusion molecules are formatted to separate the cytotoxic domain from the TAT fragment inside infected cells, thereby

avoiding unnecessary concentration of the protein in the nucleus or with RNA. It is recognized that in uninfected cells, such fusion proteins may be directed to the nucleus or to RNA. Thus, differential localization of the fusion protein in infected and non-infected cells can provide means of distinguishing such cells from one another, e.g., by inspection.

The anti-pathogen system of the invention can also positively impact certain drug-based anti-pathogen therapies. More specifically, cells infected by retroviruses and particularly HIV can harbor infectious particles for long periods of time, sometimes months or even years. Over this time, retroviruses can develop substantial resistance to most drugs, sometimes by changing one or only a few genomic sequences. It has been recognized that once the retroviruses become resistant to one class of drugs, such viruses can be become resistant to a spectrum of drugs. Thus, therapies using drug-based approaches are generally inflexible and do not readily adapt to presence of resistant viruses. Related concerns have been raised with respect to development of other resistant pathogen strains such as certain plasmodia.

In contrast, the present anti-pathogen systems kills or injures cells infected by pathogens regardless of pathogen capacity to acquire drug resistance. It is believed that development of drug resistant pathogens and particularly drug resistant HIV strains, is nearly impossible with the present anti-pathogen system due to the large number of protease cleavage sites that the system can accommodate. As an illustrative example, HIV virus has been reported to have about 8 to 10 such cleavage sites. In order to develop substantial resistance against the anti-pathogen system, which system could include one or more of these sites, that virus would have to modify those cleavage sites as well as the corresponding viral protease.

Accordingly, use of the present anti-pathogen system is expected to significantly reduce or even eliminate the presence of many pathogen resistant strains and particularly certain drug resistant HIV strains.

Additionally, the anti-pathogen system of the invention is compatible with a variety of drug-based therapies. Thus, the anti-pathogen system can be used as a sole active agent or in combination with one or more therapeutic drugs, e.g. to minimize or eliminate pathogens and particularly drug resistant pathogen strains.

Further provided are substantially pure fusion molecules of the invention.

The invention also provides nucleic acid sequences encoding the fusion proteins, particularly extrachromosomal DNA sequences organized as an autonomously replicating DNA vector.

The invention also provides methods for suppressing or eliminating infection by one or more pathogens in a mammal, particularly a primate such as a human. The methods more specifically include administering a therapeutically effective amount of the present anti-pathogen system. The methods further include treatment of a mammal that suffers from or is susceptible to infection by one or pathogens.

Preferred methods according to the invention for suppressing or eliminating infection by the one or more pathogens include providing the anti-pathogen system as an aerosol and administering same, e.g., through nasal or oral routes. Particularly contemplated are modes of administration which are specifically designed to administer the anti-pathogen system to lung tissue so as to facilitate contact with lung epithelia and enhance transfer into the bloodstream.

Also provided are methods of inducing apoptosis in a pre-determined population of cells in which the method comprises administering to the mammal such as a primate and particularly a human a therapeutically sufficient amount of the anti-pathogen system in the presence of one more pathogens.

The cell infected by one or more pathogens may be a cell maintained in culture, e.g., an immortalized cell line or primary culture of cells or tissue; or the cell can be part of a tissue or organ *in vivo* (e.g., lung). Thus, the present anti-pathogen system can be used *in vitro* and *in vivo* as needed.

The invention also provides substantially pure fusion molecules and particularly fusion proteins that in addition to the aforementioned transduction and cytotoxic domains may also include other components as needed. These components can be covalently or non-covalently linked thereto and may particularly include one or more polypeptide sequences. An added polypeptide sequence will sometimes be referred to herein as protein identification or purification "tag". Exemplary of such tags are EE, 6Xhis, HA and MYC.

As discussed, it is preferred that the fusion proteins described herein by provided in misfolded form although in some instances it may be desirable to use properly folded fusion proteins. The misfolded fusion proteins are typically purified by chromatographic approaches that can be tailored if needed to purify a desired fusion molecule from cell components which naturally accompany it. Typically, the approaches involve isolation of inclusion bodies from suitable host cells, denaturation of misfolded fusion proteins, and use of conventional chromatographic methods to purify the fusion molecules. Expression of the misfolded fusion proteins in the inclusion bodies has several advantages including protecting the misfolded fusion protein from degradation by host cell proteases. In addition, by providing the fusion proteins in misfolded form, time-consuming and costly protein refolding techniques are avoided.

Further provided by the present invention are methods of making substantial quantities of the fusion molecules and especially the fusion proteins disclosed. Generally stated, the methods include expressing

desired fusion molecules in suitable host cells, culturing the cells, and purifying the fusion molecules therefrom to obtain substantially pure fusion molecules. The methods can be used to express and purify a desired fusion protein on a large-scale (i.e. in at least milligram quantities) from a variety of implementations including roller bottles, spinner flasks, tissue culture plates, bioreactor, or a fermentor.

For fusion molecules of this invention which include certain linked molecules such as synthetic or semi-synthetic drugs, those fusion molecules can be readily made and purified if desired by one or a combination of standard approaches.

The present methods for isolating and purifying the fusion proteins of the invention are highly useful. For example, for a fusion protein exhibiting a desired killing or injuring activity, it is very useful to have methods for expressing and purifying the fusion proteins. It is particularly useful to have methods that can produce at the fusion proteins in large quantities, so that the fusion molecule can be made as one component of a kit suitable for medical, research, home or commercial use. Further, it is useful to have large-scale quantities of the fusion proteins available to simplify structural analysis, as well as for further purification and/or testing if desired.

The invention also features *in vitro* and *in vivo* screens to detect compounds with therapeutic capacity to modulate and preferably inhibit, proteins and especially proteases induced by a pathogen infection. For example, one method generally comprises infecting a desired cell with a pathogen, contacting the cell with a fusion protein of the invention, transducing the fusion protein, adding the compound to the cells and detecting cells killed or injured by the fusion protein. Efficacy of a particular compound can be readily evaluated by determining the extent of cell killing or injury as a function of concentration of the added compound.

Further provided are methods of suppressing a pathogen infection in a mammal, particularly a primate such as a human, comprising administering to the mammal a therapeutically effective amount of the anti-pathogen system. In one embodiment, the fusion protein includes a covalently linked protein transduction domain and a cytotoxic domain. The method includes transducing the fusion protein into cells of the mammal, cleaving the fusion protein sufficient to release the cytotoxic domain from the fusion protein, concentrating the cytotoxic domain in the cells; and producing a cytotoxin sufficient to suppress the pathogen infection in the mammal. Exemplary pathogens include but are not limited to retroviruses, herpesviruses, viruses capable of causing influenza or hepatitis; and plasmodia capable of causing malaria. Preferred cytotoxic domains and cytotoxins are described in more detail below.

In another embodiment of the method for suppressing the pathogen infection in the mammal, a prodrug is administered (e.g., a suitable nucleoside or analog thereof) and a cytotoxin is produced by contacting the prodrug with the concentrated cytotoxic domain.

Further provided by the present invention are fusion proteins that include covalently linked in sequence: 1) A TAT segment and particularly a protein transducing fragment thereof, and 2) a pathogen induced or host cell induced protease, e.g., HIV protease; or a catalytically active fragment thereof.

Additionally provided by the invention is an anti-pathogen system, wherein the fusion protein comprises covalently linked in sequence: 1) a transduction domain, 2) a first zymogen subunit, 3) a protease cleavage site, and 4) a second zymogen subunit. Also provided is an anti-pathogen system, wherein the transduction domain is TAT, the first zymogen subunit is p5 Bid, the protease cleavage site is an HIV protease cleavage site and the second zymogen subunit is p15 Bid.

The invention also provides an anti-pathogen system, wherein the fusion protein comprises covalently linked in sequence: 1) a transduction domain, 2) a first protease cleavage site, 3) first zymogen subunit, 3) a second protease cleavage site, and 4) a second zymogen subunit. Also provided is an anti-pathogen system, wherein the transduction domain is TAT, the first protease cleavage site is an HIV p7-p1 protease cleavage site, the first zymogen subunit is p17 caspase-3, the second protease cleavage site is an HIV p17-p24 protease cleavage site, and the second zymogen subunit is p12 caspase-3.

Also provided are novel fusion molecules which include covalently linked in sequence: 1) a transduction doman and especially at least one and preferably one PTD; and 2) at least one linked molecule and preferably one of a vaccine, steroid or peptide hormone, anesthetic, analgesic, anti-inflammatory, anti-infectious agent, anti-tumor drug, cardiovascular drug, vitamin, drug with pharmacological activity in the nervous system especially the peripheral nervous system (PNS) and central nervous system (CNS), or a diagnostic marker.

Additionally provided are novel fusion molecules that include covalently linked in sequence: 1) a transduction doman and especially at least one and preferably one PTD; 2) at least one and preferably one di-sulfide linkage; and 3) at least one linked molecule, preferably one of a vaccine, steroid or peptide hormone, anesthetic, analgesic, anti-inflammatory, anti-infectious agent, anti-tumor drug, cardiovascular drug, vitamin, drug with pharmacological activity in the nervous system especially the peripheral nervous system (PNS) and central nervous system (CNS), or a diagnostic marker.

Additionally provided are novel fusion molecules that include covalently linked in sequence: 1) at least one transduction doman and especially at least one and preferably one PTD; 2) at least one and preferably one linker sequence; and 3) at least one linked molecule, preferably one of a vaccine, steroid or peptide hormone, anesthetic,

analgesic, anti-inflammatory, anti-infectious agent, anti-tumor drug, cardiovascular drug, vitamin, drug with pharmacological activity in the nervous system especially the peripheral nervous system (PNS) and central nervous system (CNS), or a diagnostic marker.

In a particular embodiment, the linker sequence of the fusion molecule can include at least one and preferably one cleavable di-sulfide linkage. Alternatively, or in addition, the linker sequence can include at least one and preferably between from about 1 to 5 and typically about 1 cleavage site for a cell protease sometimes referred to herein as a "housekeeping" protease. Alternatively, the linker sequence can include cleavage sites for other proteases including those activated by a specific disease condition.

The present invention also provides a method of killing an HIV-infected cell. In one embodiment, the method includes contacting the cell with an effective dose of a fusion protein, wherein the fusion protein comprises covalently linked in sequence: 1) a transduction domain, 2) a first zymogen subunit, 3) a protease cleavage site, and 4) a second zymogen subunit; or 1) a transduction domain, 2) a first protease cleavage site, 3) first zymogen subunit, 3) a second protease cleavage site, and 4) a second zymogen subunit. The fusion protein can be administered *in vitro* or *in vivo* as needed. For example, the fusion protein can be administered in vivo to a mammal in need of such treatment, e.g., a primate and particularly a human patient infected by the HIV virus.

Reference herein to a "Blue" mouse or related term is meant to denote illustrative transduction of a protein fusion that includes at least the active portion of a bacterial β-gal enzyme and preferably the entire enzyme from *E. coli.* See Example 17 below. It is believed that the "Blue" mouse exemplifies the suprising power and capacity of the present invention to detectably transduce an entire living mammal including rodents such as mice and rats, rabbits, primates and

especially humans. The "Blue" mouse particulary shows that it is possible to introduce very large proteins into the brain and other tissues as essentially intact and biologically active molecules. It will be appreciated that the term "Blue" mouse not only references a particular mouse having essentially global transduction of the beta-gal enzyme. The term is also meant to reference a mouse having essentially global transduction of any other suitable molecule as disclosed herein including vaccines, drugs, and agents having or suspected of having pharmacological activity.

In accord with this invention, transduced proteins can then be analyzed in the Blue mouse in one or more specific organs, tissues and cell types, e.g, the brain or related tissues. Alternatively, the transduced proteins can be studied in the whole animal to detect more global effects, e.g., immunotolerance including graft rejection; behavior including intelligence, spatial recognition and the like; as well as functions particular to nervous system function, e.g., vision, taste, reflex including touch, hearing, and other motor or sensory parameters.

As will be discussed in detail below, the "Blue" mouse of this invention has several important uses including providing a convenient whole animal model for screening transduction molecules of interest and particularly to detect and characterize pharmacological activity of the molecule.

Other aspects of the invention are disclosed *infra.*

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a plasmid map of pTAT/pTAT-HA.

Figure 2 shows nucleotide and amino acid sequences of pTAT linker and pTAT HA linker. A minimal TAT domain is in bold. Underlined sequence designates the minimal TAT domain flanked by glycine residues.

Figures 3A-D are drawings depicting illustrative DNA vectors according to the invention based on the pTAT/pTAT-HA plasmid. (3A) TAT-HSV TK fusion protein vector, (3B) TAT-Caspase-3 fusion protein vector, (3C) TAT- p16 fusion protein vector, and (3D) PTD-p16 fusion vector. Boxes designated HIS denotes optional addition of a 6XHIS tag; protein transduction domain (PTD); HIV protease-RT cleavage site (HIV_1), herpes simplex virus thymidine kinase (HSV TK); large caspase-3 domain (Lg); small caspase-3 domain (Sm); HIV p17-p24 protease cleavage site (HIV_2); p16 (mutant or wild-type p16 protein). Approximate molecular weights of the vectors are noted.

Figure 4 is a schematic drawing outlining cell killing with a fusion protein comprising an enzyme capable of converting a prodrug into an active drug. $HIV_{1,2}$ is defined in Figs. 3A-D above.

Figure 5 is a schematic drawing showing one method of constructing a TAT-CPP32 fusion protein according the invention.

Figures 6A is a bar graph showing percentage of viable cells after transduction of various TAT fusion proteins and treatment with anti-HIV drug.

Figure 6B is a table showing percentages of viable cells (under column 2) used in the bar graph of Figure 6A.

Figure 7 is a drawing showing helical wheel projections of preferred transduction proteins of this invention. "TAT (47-57)" refers to amino acids 47 to 57 of the TAT peptide (SEQ ID. NO. 2). "SFD" refers to specified transduction domain sequences. The term "relative intracellular concentration" in Figure 7 refers to the intracellular amount of transduced peptide sequence relative to the TAT peptide.

Figures 8A-C are drawings illustrating various protein constructs. Figure 8A is a diagram of the Bid protein highlighting the p5 and p15 domains. The casapase cleavage site at Arg^{59} is shown. Figure 8B outlines the cloning of the TAT-p5-HIV-p15 fusion protein. Figure 8C shows the TAT-HIV-p15 fusion protein. See text for a description of primer designations. Hatched box= overhang between primers 2R and 2F representing HIV cleavages.

Figure 9A-E are drawings showing generation and transduction of TAT fusion proteins. Figure 9A shows the caspase 3 (Casp3) protein and various TAT/HIV fusion proteins made using the Casp3 p17 and p12 domains. Figures 9B-E are graphs showing FACS analysis of various fluorescein (FITC) labeled TAT fusion proteins.

Figures 10A-B are representations of immunoblots showing in vivo processing of various TAT fusion proteins in Jurkat T cells. The immunoblots were probed with anti-p16 (Figure 10A) or anti-Caspase-3 antibody (Figure 10B).

Figures 11A-B are graphs showing activation of TAT-Casp3 and apoptotic induction in cotransduced cells. Figure 11A shows cell viability following transduction with various TAT fusion proteins along with the HIV protease inhibitor Ritonavir (Rit). Figure 11B illustrates cell viability following transduction with various TAT fusion proteins.

Figure 12A-B are graphs showing HIV protease activates TAT-$Casp3^{wt}$ protein. Figure 12A shows results of TUNEL positive cells (apoptotic end-marker) using a TAT fusion protein. Figure 12 B shows results of a caspase-3 enzyme assay using a TAT fusion protein.

Figure 13 is a graph illustrating specific killing of HIV infected cells.

Figure 14 is a schematic diagram of a mouse being injected with a fusion molecule of this invention.

Figures 15A-B are graphs showing IP injection of Cdk2-DN-FITC into a mouse. 15A) blood; 15B) splenocytes.

Figures 16A-16B are graphs showing analysis of IP injection of STD-4-FITC into a mouse. 16A) blood, 16B) splenocytes.

Figures 17A-17D are representations of photomicrographs showing confocal pictures of 25 minutes post-IP injection of the brain and quadriceps muscle.

Figures 18A-B are graphs showing characterization of TAT-FITC in vitro and in vivo.

Figures 19A-D are confocal pictures showing TAT-FITC characterization in vivo.

Figures 20A-E show a preferred fusion protein diagram (20A), pictorial (20B) and graphical (20C-E) results of transduction of TAT-beta gal in cultured cells.

Figures 21A-B are photographs showing transduction of TAT-beta-gal into mice.

Figures 22A-C are photographs demonstrating transduction of TAT-beta-gal across the blood brain barrier.

Figures 23A-C are photographs showing various aspects of the predicted TAT transduction domain structure.

Figure 24 shows several predicted helical wheel diagrams of TAT and non-naturally occuring Protein Transduction Domains (PTDs).

Figures 25A-C are graphs (25A,C) and confocal pictures (25B) showing characterization of PTD-FITC peptide in vitro.

Figure 26A is a graph and Figure 26 B is a confocal picture showing characterization of PTD-4-GFP protein in vivo.

Figure 27A-B show characterization of PTD-4-GFP protein in vitro and in vivo.

Figure 28A is a drawing showing the PTD-B-gal fusion construct.

Figure 28B is a graph showing rapid transduction, but slow refolding of a bacterial PTD-B-Gal protein.

DETAILED DESCRIPTION OF THE INVENTION

As discussed, the invention relates to novel fusion molecules that preferably include a synthetic protein transduction domain (PTD) and a linked molecule, e.g., a vaccine, steroid or peptide hormone, anesthetic, analgesic, anti-inflammatory, anti-infectious agent, anti-tumor drug, cardiovascular drug, vitamin, drug with pharmacological activity in the nervous system especially the peripheral nervous system (PNS) and central nervous system (CNS), or a diagnostic marker. Also provided are "macro-transduction" methods that allow transduction of the fusion molecule or group of such molecules into an organ up to essentially an entire subject mammal. Further provided are "micro-transduction" methods allowing transduction of the fusion molecule or group of such molecules into pre-determined sites in the mammal including one or a few cells or tissue in the mammal. As also discussed, the micro-transduction methods of this invention can be used to remove undesired cells or tissues and is often referred to herein as "micro-surgery" or a related term.

By the term "fusion molecule" as it is used herein is meant a transducing molecule that preferably includes at least one synthetic or semi-synthetic PTD, preferably between from about 1 to 5 PTDs, and more preferably one PTD, fused to at least one linked molecule of interest, preferably from between about 1 to 10 of such molecules and typically one of such molecules. That fusion can be achieved by any acceptable route such as by standard recombinant, chemical or other suitable method. If desired, the fusion molecule can include a fused peptide linker (at one or several sites) which sequence preferably bridges the PTD to the linked molecule. For use with a subject mammal including humans, the fusion molecules of this invention are preferably provided as sterile formulations.

As discussed, the fusion molecules of this invention can include nearly any linked molecule (known or yet to be discovered) provided that the molecule can be fused to a peptide or protein sequence using conventional manipulations. Methods for fusing a wide spectrum of molecules to amino acid sequences, e.g., by chemical crosslinking with the N- terminus, C-terminus, or reactive amino groups of a peptide or protein sequence are well known. Specific linkage between the molecule of interest and one end of the PTD is often preferred.

By way of illustration, see the Examples below which provide for specific fusion between one end of a PTD, e.g, PTD NO. 4 (see Table 2 below) and the fluorescent molecule FITC. See Means, G.E. and Feeney, R.E. (1974), *infra*, and S.S. Wong (1991), *infra*; and references cited therein for disclosure relating to linking molecules to amino acid sequences.

As discussed, certain fusion molecules disclosed herein include at least one peptide linker, typically one of such linkers, fused between the PTD and the linker molecule. In this embodiment, the linker can include one or more sites for cleavage, e.g., by a pathogen induced or host cell induced protease. Alternatively, or in addition, the peptide linker can

include at least one disulfide linkage and/ or at least one site for cleavage by a housekeeping protease. Typically, the peptide linker will include one disulfide linkage and one protease site. For fusion molecules that include a cytotoxic domain, that domain will usually include one potentially toxic molecule such a zymogen sometimes from between about 2 up to about 5 to 10 of such molecules.

For some applications, will not include any cleavable site and may be used to assist in construction of the fusion molecule.

A wide variety of peptide linker sequences are suitable for use with the present invention and are disclosed below.

It will be understood that cleavage of the fusion molecules of this invention by cells, tissue, organs, ect., can also be achieved without including the peptide linker sequence in the fusion molecule. As an illustration, it is possible to provide a cleavable disulfide linkage by adding a desired PTD along lines shown in the Examples below. In this instance, significant amounts of FITC are released in transduced cells following transduction and contact with cytoplasm.

Illustrative fusion molecules of this invention include the linked molecules discussed herein including specific fusion proteins provided below. Particular linked molecules of interest include those which are suspected of or have recognized activity for the treatment or prevention of a recognized medical condition afflicting human patients. Molecules suspected of or having veterinary use are also within the scope of this invention. Although this invention is particularly applicable for administering compounds having or suspected of having poor bioavailability, it will be appreciated that the invention can also be used with those compounds demonstrating acceptable bioavailability as needed.

More particular linked molecules include known agents, drugs, medicaments and other formulations that can be linked to a peptide or protein by standard cross-linking procedures as discussed previously. Examples of such compounds can be found in the *Physician's Desk Reference* (1997) Medical Economics Co, e.g., pages 203-220; and Gilman et al. *supra* for disclosure relating to these compounds.

As discussed, especially preferred fusion molecules include linked molecules which are suspected to have or have recognized capacity to treat or prevent an acknowledged medical condition such as cancer, infection, pain, gastrointestinal ailment, hormone related disease, immune disorder including graft rejection, or nervous system disorder such as Alzheimer's disease, Huntington's disease, Parkinson's disease, pre-senile dementia, age-related memory loss and Lou Gehring's disease. See the *Physician's Desk Reference* and Gilman et al. *supra* for specific examples of such molecules.

The fusion molecule can include a linked molecule already part of the medical or veterinary armamentarium, or the linked molecule can be an experimental drug such as those routinely coming out of on-going drug discovery and screening programs.

Preferred anti-infective drugs will have or be suspected of having significant capacity to treat a medical or veterinary condition, particularly a disorder impacting a human patient. Illustrative drugs inhibit the growth, proliferation or reproductive capacity of pathogenic virus, yeast, bacteria, fungi, protozoa, or nematodes.
See Gilman et al. *supra*. More particular examples include anti-biotics, vitamins, drugs whose mode of action is preferably in the CNS or PNS, autacoids, cardiovascular drugs such as anti-hypertensive agents and anti-arrhythmic drugs, anti-cancer drugs, immunosuppressive agents, ect.

As noted above, the present invention also features an anti-pathogen system that exhibits high transduction efficiency and specifically kills or injures cells infected by one or more pathogens. The anti-pathogen system generally includes a fusion protein that includes a transduction domain fused to a cytotoxic domain as a genetic in-frame fusion protein. Preferred fusion proteins exhibit enhanced transduction efficiency as determined, e.g., by assays which follow. The transduction domain transduces the fusion protein into cells and once inside the cells, the cytotoxic domain is released from the fusion protein and forms a cytotoxin in the infected cells. In preferred embodiments, function of the fusion protein has been specifically enhanced, e.g., by optimizing transduction domain structure and by misfolding the fusion molecule.

Specific fusion proteins of this invention and particularly those suited for use with the anti-pathogen system are preferably capable of killing at least about 25%, 40%, 50%, 60%, or 70%, preferably 80%, 90%, and more preferably at least 95% up to 100% of the cells infected by the pathogen as assayed by standard cell viability tests discussed below.

An "anti-pathogen system" according to the invention includes one or more of the fusion molecules described herein as well as any additional components which may be added thereto such as those which may facilitate solublization, stability and/or activity including transduction efficiency. Examples include but are not limited to a serum protein such as bovine serum albumin, a buffer such as phosphate buffered saline, or a pharmaceutically acceptable vehicle or stabilizer. See generally *Remington's Pharmaceutical Sciences*, *infra*, for a discussion of pharmaceutically acceptable vehicles, stabilizers, ect. A preferred anti-pathogen system includes from between about 1 to 3 and are preferably 1 fusion protein dissolved in a pharmaceutically acceptable carrier such as water or buffered saline. Preferably, the anti-pathogen system is provided sterile.

Additional disclosure relating to this invention including the fusion molecules and anti-pathogen system can be found in co-pending U.S. Provisional Application Serial No.60/082,402 filed on April 20, 1998, U.S. Provisional Application Serial No. 60/069,012, filed on December 10, 1997 and in the unpublished PCT application PCT/US98/26358. Additionally related disclosure can be found in the co-pending Provisional Application Serial No. 60/083,380 filed on April 28, 1998. The disclosures of each of these applications is incorporated herein by reference.

See also Vocero-Akbani, A.M. et al. (1999) *Nat. Med.* 5: 29; and Nagahara, H. et al. (1998) *Nat. Med.* 4: 1449 for additional information relating to the present invention the disclosures of which are incorporated herein by reference.

As will be discussed in more detail below, the anti-pathogen system can be administered as a sole active agent or in combination with one or more medicaments such as those specifically provided below.

The fusion molecules of this invention including specific fusion proteins disclosed herein can be organized in nearly any fashion provided that the fusion molecule has the function for which it was intended. For example, each component of the fusion proteins of this invention can be spaced from another component by at least one suitable peptide linker sequence if desired . Additionally, the fusion proteins may include tags, e.g., to facilitate identification and/or purification of the fusion protein. More specific fusion proteins are described below. More particular organization will be guided by maximization of release of linked molecules inside targeted cells, tissues and organs of the subject mammal.

Preferred peptide linker sequences typically comprise up to about 20 or 30 amino acids, more preferably up to about 10 or 15 amino acids, and still more preferably from about 1 to 5 amino acids. The linker

sequence is generally flexible so as not to hold the fusion molecule in a single rigid conformation. The linker sequence can be used, e.g., to space the DNA binding protein from the fused molecule. Specifically, the peptide linker sequence can be positioned between the protein transduction domain and the cytotoxic domain, e.g., to chemically cross-link same and to provide molecular flexibility.

The term "misfolded" as it relates to the fusion proteins is meant a protein that is partially or completely unfolded (i.e. denatured). A fusion protein can be partially or completely misfolded by contact with one or more chaotropic agents as discussed below. More generally, misfolded fusion proteins disclosed herein are representative of a high Gibbs free energy (ΔG) form of the corresponding native protein. Preferred are misfolded fusion proteins that are fully soluble in aqueous solution. In contrast, a native fusion protein is usually correctly folded, it is fully soluble in aqueous solution, and it has a relatively low ΔG. Accordingly, that native fusion protein is stable in most instances.

It is possible to detect fusion protein misfolding by one or a combination of conventional strategies. For example, the misfolding can be detected by a variety of conventional biophysical techniques including optical rotation measurements using native (control) and misfolded molecules. As noted, preferred administration of the anti-pathogen system involves transduction of misfolded fusion proteins *in vitro* and *in vivo*. Without wishing to be bound to theory, it is believed that after transduction of the fusion protein into cells, misfolded fusion proteins are significantly refolded, e.g., by chaperonens, sufficient to produce a fusion protein than can be activated in response to pathogen infection.

See the co-pending Provisional Application Serial No. 60/083,380 for additional information relating to making and using misfolded fusion molecules.

As discussed in the Provisional Application Serial No. 60/083,380, a misfolded protein is defined herein as one without one or more of

significant enzyme activity, circular dichroism, or other biochemical alteration in the fusion molecule. Of course, that biochemical alteration will depend on the specific fusion molecule of interest.

By the term "fully soluble" or similar term is meant that the fusion molecule and particularly a fusion protein that is not readily sedimented under low G-force centrifugation (e.g. less than about 30,000 revolutions per minute in a standard centrifuge) from an aqueous buffer, e.g., cell media. Further, the fusion molecule is soluble if the it remains in aqueous solution at a temperature greater than about 5-37°C and at or near neutral pH in the presence of low or no concentration of an anionic or non-ionic detergent. Under these conditions, a soluble protein will often have a low sedimentation value e.g., less than about 10 to 50 svedberg units.

Aqueous solutions referenced herein typically have a buffering compound to establish pH, typically within a pH range of about 5-9, and an ionic strength range between about 2mM and 500mM. Sometimes a protease inhibitor or mild non-ionic detergent is added. Additionally, a carrier protein may be added if desired such as bovine serum albumin (BSA) to a few mg/ml. Exemplary aqueous buffers include standard phosphate buffered saline, tris-buffered saline, or other well known buffers and cell media formulations.

A "polypeptide" refers to any polymer preferably consisting essentially of any of the 20 natural amino acids regardless of its size. Although the term "protein" is often used in reference to relatively large proteins, and "peptide" is often used in reference to small polypeptides, use of these terms in the field often overlaps. The term "polypeptide" refers generally to proteins, polypeptides, and peptides unless otherwise noted.

By the term "potentially toxic molecule" is meant an amino acid sequence such as a protein, polypeptide or peptide; a sugar or

polysaccharide; a lipid or a glycolipid, glycoprotein, or lipoprotein that can produce the desired toxic effects as discussed herein. Also contemplated are potentially toxic nucleic acids encoding a toxic or potentially toxic protein, polypeptide, or peptide. Thus, suitable molecules include regulatory factors, enzymes, antibodies, or drugs as well as DNA, RNA, and oligonucleotides. The potentially toxic molecule can be naturally-occurring or it can be synthesized from known components, e.g., by recombinant or chemical synthesis and can include heterologous components. A potentially toxic molecule is generally between about 0.1 to 100 KD or greater up to about 1000 KD, preferably between about 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 ,30 and 50 KD as judged by standard molecule sizing techniques such as centrifugation or SDS-polyacrylamide gel electropheresis.

As used herein, the term "cell" is intended to include any primary cell or immortalized cell line, any group of such cells as in, a tissue or an organ. Preferably the cells are of mammalian and particularly of human origin, and can be infected by one or more pathogens. A "host cell" in accord with the invention can be an infected cell or it can be a cell such as *E. coli* that can be used to propagate a nucleic acid described herein.

The present anti-pathogen system is suitable for *in vitro* or *in vivo* use with a variety of cells that are infected or that may become infected by one or more pathogens.

As an illustration of the use of the anti-pathogen system, a cultured cell can be infected by a pathogen of a single serotype. The infected cell is then contacted by a specified fusion protein *in vitro*. As discussed previously, the fusion protein is configured so that the cytotoxic domain is activated in the presence of one or more proteases induced by the pathogen infection. After providing for transduction into the cell (generally less than about 30 minutes), the cells are allowed to cleave the fusion protein for a time period of about up to about 2 to 24 hours, typically about 18 hours. After this time, the cells are washed in

a suitable buffer or cell medium and then evaluated for viability. The time allotted for cell killing or injury by the fusion protein will vary with the particular cytotoxic domain chosen. However viability can often be assessed after about 2 to 6 hours up to about 24 hours. As will be explained in more detail below, cell viability can be readily measured and quantified by monitoring uptake of certain well-known dyes (e.g., trypan blue) or fluors.

As noted above, the anti-pathogen system is flexible and can be provided in formats that are tailored for a specific use. For example, the system can be provided with two fusion proteins in which the first fusion protein includes a transduction domain and a cytotoxic domain, and the second fusion protein includes a transducing domain and a pathogen-induced or host cell induced protease.

Cells transduced by the fusion molecules of the present invention can be assayed for viability by standard methods. In one approach, cell viability can be readily assayed by measuring DNA replication following or during transduction. For example, a preferred assay involves cell uptake of one or more detectably-labeled nucleosides such as radiolabelled thymidine. The uptake can be conveniently measured by several conventional approaches including trichloroacetic acid (TCA) precipitation followed by scintillation counting. Other cell viability methods include well know trypan blue exclusion techniques.

As noted, fusion molecules of the present invention are efficiently transduced into target cells or groups of such cells. Transduction efficiency can be monitored and quantified if desired by one or a combination of different strategies.

For example, one approach involves an *in vitro* assay that measures uptake of the fusion protein by the cell. The assay includes detectably-labeling the fusion protein with, e.g., a radioactive atom, fluorescent, phosphorescent, or luminescent tag (e.g., fluorescein,

rhodamine or FITC) and then measuring uptake of the labeled fusion protein. Alternatively, the fusion protein can be labeled with an enzyme capable of forming a detectable label such as horseradish peroxidase, β-galactosidase, chloramphenicol acetyl transferase or luciferase. In a preferred approach, it is possible to genetically fuse a desired fusion protein to the well-known green fluorescent protein (GFP) and then assaying the fusion protein. Uptake can be measured by several conventional methods such as by quantifying labeled cells in a standard cell sorter (e.g., FACS), by fluorescence microscopy or by autoradiography. See generally Sambrook et al. and Ausubel et al. *infra* for disclosure relating to the assays.

Preferred fusion proteins of the invention are capable of transducing at least about 20%, to 80%, and more preferably at least about 90%, 95%, 99% up to 100% of the total number of target cells as determined by any conventional methos for monitoring protein uptake by cells and particularly the FACS or related microscopical techniques. The total number of target cells can be estimated by standard techniques.

As noted, the present invention pertains to fusion proteins and nucleic acids (e.g., DNA) encoding the fusion proteins. When the cytotoxic domain is a polypeptide sequence, the term fusion protein is intended to describe at least two polypeptides, typically from different sources, which are operatively linked. With regard to the polypeptides, the term "operatively linked" is intended to mean that the two polypeptides are connected in manner such that each polypeptide can serve its intended function. Typically, the two polypeptides are covalently attached through peptide bonds. As discussed, the two polypeptides may be separated by a peptide linker if desired.

The fusion proteins described herein are preferably produced by standard recombinant DNA techniques. For example, a DNA molecule encoding the first polypeptide can be ligated to another DNA molecule encoding the second polypeptide. In this instance, the resultant hybrid

DNA molecule can be expressed in a suitable host cell to produce the fusion protein. The DNA molecules are ligated to each other in a 5' to 3' orientation such that, after ligation, the translational frame of the encoded polypeptides is not altered (i.e., the DNA molecules are ligated to each other in-frame). The resulting DNA molecules encode an in-frame fusion protein.

The components of the fusion protein can be organized in nearly any order provided each is capable of performing its intended function. For example, in one embodiment, the protein transduction domain is adjacent to a pathogen-specific protease cleavage site included within the cytotoxic domain. Additionally, the cytotoxic domain can be flanked by pathogen-specific protease cleavage sites, one or both of which can also be adjacent to the protein transduction domain. The present invention also contemplates circular fusion proteins.

Preferred cytotoxic domains including the pathogen-specific cleavage sites will have sizes conducive to the function for which those domains are intended. In particular, preferred cytotoxic domains can be at least about 0.1, 0.2, 0.5, 0.75, 1, 5, 10, 25, 30, 50, 100, 200, 500 kD, up to about 1000 kD or more. It should be apparent that the size of the cytotoxic domain usually dominates the size of the fusion protein. Preferred pathogen-specific cleavage sites will be between about 4 to about 30 or 40, preferably about 8 to about 20 and more preferably about 14 amino acids in length. See Table I, below. The pathogenic-specific protease cleavage sites can be made and fused to the cytotoxic domain by a variety of methods including well-known chemical cross-linking methods. See e.g., Means, G.E. and Feeney, R.E. (1974) in *Chemical Modification of Proteins*, Holden-Day. See also, S.S. Wong (1991) in *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press. However it is generally preferred to use recombinant manipulations to make the in-frame fusion protein.

As noted, a fusion molecule in accord with the invention can be organized in several ways. In an exemplary configuration, the C-terminus of the transduction domain is operatively linked to the N-terminus of the cytotoxic domain. That linkage can be achieved by recombinant methods if desired. However, in another configuration, the N-terminus of the transduction domain is linked to the C-terminus of the cytotoxic domain. Within the cytotoxic domain, the N-terminus of a first pathogen-specific protease cleavage site can be operatively linked to the C-terminus of the transduction domain and the C-terminus of the protease cleavage site can be operatively linked to the N-terminus of a potentially toxic molecule. In yet another configuration, the C-terminus of the cytotoxic domain can be linked to the N-terminus of a second pathogen-specific protease cleavage site the same or different from the first pathogen-specific site. Preferably, the first and second pathogen-cleavage sites will be specifically cleaved by the same protease induced by the pathogen infection.

Alternatively, or in addition, one or more additional protease cleavage sites can be inserted into the potentially toxic molecule as needed.

Preferred fusion proteins in accord with the present invention typically include operatively linked in sequence (N to C terminus): 1) a transduction domain/one or more pathogen-specific protease cleavage sites/and a potentially toxic molecule; 2) a transduction domain/a pathogen specific protease cleavage site/and a zymogen; and 3) a transduction domain/a first pathogen specific protease cleavage site/a first zymogen subunit/a second pathogen specific protease cleavage site/and a second zymogen subunit. In addition, one or more protein tags such as EE, HA, Myc, and polyhistidine, particularly 6Xhis, can be fused to the N-terminus of the transduction domains as desired, e.g., to improve solubility or the facilitate isolation and identification of the fusion protein. See Examples below.

Although generally not preferred, it is possible to operatively link a polypeptide sequence to the fusion proteins to promote transport to a cell nucleus. Amino acid sequences which, when included in a protein, function to promote transport of the protein to the nucleus are known in the art and are termed nuclear localization signals (NLS). Nuclear localization signals typically are composed of a stretch of basic amino acids. When attached to a heterologous protein (e.g., a fusion protein of the invention), the nuclear localization signal promotes transport of the protein to a cell nucleus. The nuclear localization signal is attached to a heterologous protein such that it is exposed on the protein surface and does not interfere with the function of the protein. Preferably, the NLS is attached to one end of the protein, e.g. the N-terminus. The SV40 nuclear localization signal is a non-limiting example of an NLS that can be included in a fusion protein of the invention. The SV40 nuclear localization signal has the following amino acid sequence: Thr-Pro-Pro-Lys-Lys-Lys-Lys-Arg-Lys-Val (SEQ ID NO:3). Preferably, a nucleic acid encoding the nuclear localization signal is spliced by standard recombinant DNA techniques in-frame to the nucleic acid encoding the fusion protein (e.g., at the 5' end).

As noted above, a fusion protein of the invention is composed, in part, of a first polypeptide, sometimes referred to herein as a protein transduction domain, transduction domain, transducing protein, or "PTD", which provides for entry of the fusion protein into the cell. Peptides having the ability to provide entry of a coupled peptide into a cell are known and include those mentioned previously such as TAT, Antennapedia homeodomain, referred to as "Penetratin" Ala-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-Glu-Asn (SEQ ID. NO:1) (Derossi et al., *J. Bio. Chem.*, 269:10444 (1994)) and HSV VP22 (Elliot and O'Hare, *Cell*, 88:223 (1997)).

Illustrative of a transducing protein is a TAT fragment that includes at least the TAT basic region (amino acids 49-57 of naturally-occurring TAT protein). TAT fragments can be between about 9, 10, 12,

15, 20 , 25, 30, or 50 amino acids in length up to about 86 amino acids in length. The TAT fragments preferably are deficient in the TAT cysteine-rich region (amino acids 22-36 of naturally-occurring TAT protein) and the TAT exon 2 encoded by a carboxy-terminal domain (amino acids 73-86 of naturally-occurring TAT protein). A TAT transduction domain has the following amino acid sequence: YGRKKRRQRRR (SEQ ID. NO:2). That amino acid sequence will sometimes be referenced herein as a "minimal TAT sequence". See U. S. Pat. No. 5,674,980 and references cited therein for disclosure relating to TAT structure. See also Green, M. and Lowenstein, P. M. (1988) for the TAT sequence.

Various transduction enhancing modifications of the TAT fragment are contemplated as discussed above and in the Examples which follow. For example, the protein transduction domain of the fragment can be flanked by glycine residues to allow for free rotation. See e.g., Fig. 2 of the drawings. Alternatively, other amino acid sequences and particularly neutral and/or hydrophilic residues may be added to the TAT fragment as desired. Protein tags may be added to a TAT fragment such as those known in the field. Examples of such protein tags include 6XHis, HA, EE and Myc. In general, the size of the modified TAT fragment will be at least 10, 12, 15, 20 , 25, 30, 50, 100, 200, to about 500 amino acids in length.

The transduction domain of the fusion protein can be obtained from any protein or portion thereof that can assist in the entry of the fusion protein into the cell. As noted, preferred proteins include, for example TAT, *Antennapedia* homeodomain and HSV VP22 as well as non-naturally-occurring sequences. The suitably of a synthetic protein transduction domain can be readily assessed, e.g., by simply testing a fusion protein to determine if the synthetic protein transduction domain enables entry of the fusion protein into cells as desired.

By the term "synthetic protein" or like term a non-naturally occurring amino acid sequence which is made be recombinant methods or methods involving chemical peptide synthesis.

Numerous variants of transducing TAT proteins have been described in the field. These variants can be used in accord with the present invention. See e.g., U.S. Pat. No. 5,652,122 which reports methods of making and using transducing TAT proteins, the disclosure of which is incorporated by reference.

Additional transduction domains and particularly transducing proteins can be readily identified by conventional techniques. For example, in one approach, a candidate transduction domain such as a desired TAT fragment is fused to a desired cytotoxic domain using standard recombinant manipulations to form the in-frame fusion protein. The fusion protein is subsequently detectably-labeled with, e.g., a radioactive atom or fluorescent label such as FITC. The detectably-labeled fusion protein is then added to cells as described above and the levels of the fusion protein are measured. A preferred transduction domain will be capable of achieving an intracellular concentration of the fusion protein of between about 1 picomolar to about 100 micromolar, preferably about 50 picomolar to about 75 micromolar, and more preferably about 1 to about 100 nanomolar.

Particularly contemplated transducing proteins are those obtained by targeted mutagenesis of known transducing proteins or fragments, e.g., TAT, VP22 or the *Antennapedia* homeodomain sequences mentioned above. Typically, the mutagenized transducing protein will exhibit at least about 2, 3, 4, 5, 10, 20, 30, 40 or 50 fold better transduction of a desired fusion protein when compared to that same fusion protein comprising a corresponding full-length transducing protein sequence.

Preferred transduction proteins in accord with this invention are Class I amino acid sequences, preferably peptide sequences, that include

at least a peptide represented by the following general formula: B1 -X_1-X_2-X_3-B_2-X_4-X_5-B_3; wherein B_1, B_2, and B_3 are each independently a basic amino acid, the same or different; and X_1 , X_2 , X_3 , X_4 and X_5 are each independently an alpha-helix enhancing amino acid the same or different. Typically these sequences are synthetic.

The term "basic amino acid" or like term as used herein refers to an amino acid having a basic residue such as a primary, secondary or tertiary amine, or a cyclic group containing nitrogen ring member. Preferred basic amino acids are lysine (Lys) and arginine (Arg), with arginine being particularly preferred. Histidine (His) also can be a suitable basic amino acid.

The term "alpha-helix enhancing" amino acid or like term is meant an amino acid which has a recognized tendency to form or stabilize an alpha-helix as measured by assays well-known in the field. See generally O'Neil, K.T. and DeGrado, W.F. (1990) *Science* 250: 646 and references cited therein for such an assay. Preferred alpha-helix enhancing amino acids include alanine (Ala), arginine (Arg), lysine (Lys), leucine (Leu), and methionine (Met). A particularly preferred alpha-helix enhancing amino acid is alanine. By the term "substantial alpha-helicity" is meant that a particular peptide has a recognizable alpha-helical structure as determined, e.g., by a helical wheel diagram or other conventional means.

In one embodiment, the peptide is represented by the formula B_1 -X_1-X_2-X_3-B_2-X_4-X_5-B_3; wherein at least one of B_1, B_2, or B_3 is arginine, preferably all of B_1, B_2, and B_3 is arginine; and X_1, X_2, X_3 , X_4 and X_5 are each independently an alpha-helix enhancing amino acid the same or different. Preferably at least one of X_1, X_2, X_3 , X_4 or X_5 is an alanine, more preferably all of X_1 , X_2 , X_3 , X_4 and X_5 are alanine. In another embodiment, the peptide is represented by the formula B_1 -X_1-X_2-X_3-B_2-X_4-X_5-B_3; wherein B_1, B_2, and B_3 are each independently a basic amino acid, the same or different; and at least one of X_1, X_2, X_3 , X_4 or X_5 is

alanine, preferably all of X_1 , X_2 , X_3 , X_4 and X_5 each is alanine. In these embodiments, basic amino acid residues such as arginine are substantially aligned along at least one face of the peptide, typically along one face.

Additionally preferred transduction proteins in accord with this invention are synthetic amino acid sequences, preferably peptide sequences, that include at least a peptide represented by the following general formula: B_1 -X_1-X_2-B_2 -B_3 -X_3-X_4-B_4; wherein B_1, B_2, B_3, and B_4 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3 , and X_4 are each independently an alpha-helix enhancing amino acid the same or different. In a one embodiment, at least one of B_1, B_2, B_3, or B_4 is arginine, preferably all of B_1, B_2, B_3, and B_4 are arginine; and the X_1, X_2, X_3 , and X_4 are each independently an alpha-helix enhancing amino acid the same or different.. In another embodiment, each of the B_1, B_2, B_3, and B_4 are independently a basic amino acid, the same or different; and at least one of X_1, X_2, X_3 , or X_4 is an alanine, preferably all of X_1 , X_2 , X_3 , and X_4 are alanine residues. In these embodiments, basic amino acid residues such as arginine are substantially aligned along at least one face of the peptide, typically along one face.

By the term "substantial alignment" of a basic amino acid residue or like term is meant that the basic amino acid residue is positioned with respect to at least one other basic amino acid residue so that each residue is spaced from the other on a conceptualized alpha-helix by between about 3 to about 4 Angstroms, preferably about 3.6 Angstroms. Alignment can be performed by several conventional methods including inspection of standard helical wheel diagrams such as those shown below in Figure 7. Preferred transduction domains exhibit between about 2, 3, 4, 5, 6, or about 7 up to about 10 substantially aligned basic amino acid residues.

More preferred transduction proteins of this invention include at least a peptide represented by the following specific peptide sequences:

YARKARRQARR (SEQ ID NO. 3), YARAAARQARA (SEQ ID NO. 4), YARAARRAARR (SEQ ID NO. 5), YARAARRAARA (SEQ ID NO. 6), YARRRRRRRRR (SEQ ID NO. 7), and YAAARRRRRRR (SEQ ID NO. 8). Particularly preferred are transducing peptide sequences consisting of the following peptide sequences: YARKARRQARR (SEQ ID NO. 3), YARAAARQARA (SEQ ID NO. 4), YARAARRAARR (SEQ ID NO. 5), YARAARRAARA (SEQ ID NO. 6), YARRRRRRRRR (SEQ ID NO. 7), and YAAARRRRRRR (SEQ ID NO. 8).

Additional transduction proteins of this invention are amino acid sequences, preferably synthetic sequences, that include at least one amino acid modification in at least amino acids 49 to 56 of TAT. For example, in one embodiment, the synthetic peptide sequences include at least amino acids 47 to 56, 48 to 56, 47 to 57, 48 to 57, or 49 to 57 of TAT which TAT sequence has been modified to increase the alpha-helicity of that TAT sequence relative to a suitable TAT control sequence. Preferably, the TAT sequence includes at least one amino acid substitution with an alpha-helix enhancing amino acid such as alanine.

Additional transduction proteins are amino acid sequences, preferably synthetic peptide sequences that include at least amino acids 47 to 56, 48 to 56, 47 to 57, 48 to 57, or 49 to 57 of TAT which TAT sequence has been modified so that two or more basic amino acids such as arginine are substantially aligned along at least one face of that TAT sequence. The alignment can be facilitated by a variety of approaches including visualizing the TAT sequence as an alpha-helix on a helical wheel. See Figure 7 which follows.

Further transduction proteins of this invention are peptide sequences that include at least amino acids 49 to 56 of TAT, preferably 47 to 56, 48 to 56, 49 to 56, 47 to 57, 48 to 57, or 49 to 57 of TAT, in which the TAT sequence includes at least one amino acid substitution with an alpha-helix enhancing amino acid. In this embodiment, the amino acid substitution is selected to align substantially two or more

arginine residues along at least one face of that TAT sequence, preferably alone one face of the TAT sequence. In one embodiment, preferably about 2, 3, 4, or 5 arginine residues are substantially aligned along at least one face of the helix, more specifically along one face of the helix. For example, about 1, 2, 3, 4, or 5 amino acid residues up to about 6 amino acids residues in the TAT sequence can be substituted with an alanine residue to enhance alpha-helicity and to align the arginine residues on at least one face of the helix.

Additional transduction proteins in accord with this invention include amino acid sequences that comprise at least a transducing portion of the Antp sequence (SEQ ID NO 10; see Table 2 below), preferably the Antp sequence which has been modified along lines described above for specified TAT sequences. In particular, the modifications can include at least one suitable amino acid substitution, deletion or addition that has been selected to enhance the alpha-helicity of the transduction protein, to align basic amino acid residues (e.g., Arginine) along at least one face of the Antp sequence, or both. Illustrative are transduction proteins that include the Antp sequence (SEQ ID NO. 10) in which the Antp sequence has been modified to include at least one amino acid modification sufficient to increase transduction efficiency of the protein by between about 2, 5 or 10 up to 100 or more fold compared to a suitable control peptide, e.g., the Antp sequence (SEQ ID NO. 10).

Additionally preferred are class II transducing amino acid sequence as described above. In one embodiment, the class II sequence is a peptide represented by the following formula: X_1-X_2-R X_3-(P/X_4)-(B/X_5)-B-(P/X_6)-X_4-B-(B/X_7), wherein each of X_1, X_2, X_3, X_4, X_5, X_6, X_7 is an alpha helical promoting residue the same or different; each of (P/X_4) and (P/X_6) are independently proline or an alpha helical promoting residue; B is a basic amino acid residue; (B/X_5) (B/X_7) are each independently B or an alpha helical promoting residue; and R is arginine (Arg). A preferred alpha helical promoting residue is alanine (Ala). Preferred basic

amino acid residues are arginine (Arg), lysine (Lys), especially Arg. Particularly preferred class II transducing amino acid sequences include at least one proline residue, usually between from about one to three residues.

More specifically preferred class II peptide sequences are provided in Example 13 below (SEQ ID Nos. 36-41).

Molecular weights of the transduction domains described herein will vary according to parameters such as intended use and transduction efficiency desired. Generally, the transduction domain will exhibit a molecular weight of between from about 1, 2, 3, 5, 10, 20 to about 50 kDa as judged by SDS-PAGE gel electrophoresis or other suitable assay. Specifically preferred transduction domains are described more fully below and in the examples which follow.

As noted, preferred transduction domains in accord with the present invention will exhibit enhanced transduction efficiency. That increase can be evaluated by one or a variety of standard techniques such as those specifically described below. In one general approach sometimes referred to herein as a transduction efficiency assay or similar term, the transduction efficiency is determined by reference to a control assay in which one or more suitable control molecules are transduced into cells in parallel with a desired transduction protein. Preferably, transduction rate and intracellular amounts of a specified transduction domain are measured and compared to the control molecule. Illustrative control molecules suitable include amino acids 47 to 57 of TAT (SEQ ID NO: 1), amino acids 49 to 57 of TAT, and the Antp sequence (SEQ ID NO:10).

Two or more protein transduction domains of this invention, e.g., about 2, 3, 4, 5, 6, up to about 10 or more protein transduction domains, can be covalently linked to a desired molecule to be transduced. In this

embodiment, the protein transduction domains can be linked in tandem or can be separated by at least one suitable peptide linker as desired.

Preferred transduction proteins of this invention exhibit an increase in transduction efficiency of between about 5 to 10 up to 100 or more fold when compared to a suitable control sequence, e.g., the minimal TAT sequence or other suitable control molecule. Examples of preferred transduction assays are described below in Example 7.

The transduction proteins of the present invention can be made by a variety of conventional methods. For example, DNA coding for a desired transduction protein can be obtained by isolating DNA from natural sources or by known synthetic methods, e.g. the phosphate triester method. See, e.g., *Oligonucleotide Synthesis*, IRL Press (M. Gait, ed., 1984). Synthetic oligonucleotides also may be prepared using commercially available automated oligonucleotide synthesizers. The synthetic oligonucleotide encoding the transducing protein may be inserted into a variety of suitable vectors (e.g., pTAT Vector) and expressed in an appropriate host cell. See generally Ausubel et al. and Sambrook et al. *infra*.

As discussed, components of the fusion proteins can be linked in several ways. In one embodiment, the transduction domain of the fusion protein is operatively linked to a cytotoxic domain (sometimes referred to herein as "CD"). As also discussed, the function of the cytotoxic domain in this example is to produce a cytotoxin that can kill or injure infected cells under specified conditions. The cytotoxic domain is transduced into the cell as part of the fusion molecule, and it is specifically intended to be released from that fusion molecule in the presence of one or more specified proteases induced by the pathogen infection. In some instances, release of the cytotoxin will be accompanied by further processing or maturation by the hosting cell. A preferred method of operatively linking the transduction domain and the cytotoxic domain is

to use a nucleic acid sequence which encodes same ligated together to form an in-frame genetic fusion protein.

As noted previously, the present invention is compatible with a variety of cytotoxic domains. Preferred cytotoxic domains include potentially toxic molecules such as known zymogens. In general, most zymogens exhibit insignificant catalytic activity. However, once activated by protein modification and particularly proteolysis at one or more protease cleavage sites, the zymogens are converted into mature enzymes. In instances where the conversion (maturation) includes proteolysis, the cleavage occurs at site specific locations in the zymogen. Sometimes fragments released from the zymogen are themselves catalytically active and upon release, further process the immature enzyme to a less immature or fully mature form. Zymogens including such fragments are often referred to as autocatalytic enzymes. In other cases however, the fragments are devoid of significant catalytic activity and must be cleaved to form the mature enzyme. A particular catalytic fragment can be naturally-associated with the zymogen or it can be recombinantly added to zymogen in accord with standard techniques to form a heterologous zymogen. Naturally-occuring protease cleavage sites in the zymogen usually serve to demarcate subunits within the zymogen. These can be replaced or added to in accordance with methods discussed herein.

Particular zymogens for use in accord with this invention include those associated with apoptosis, particularly cysteinyl aspartate-specific proteinases (caspases) and particularly caspase-3 (CPP32, apopain, Yama), caspases-5 (ICE_{rel}-III, TY), caspase-4(ICE_{rel}-II TX, ICH-2), caspase-1 (ICE), caspase-7 (Mch3, ICE-LAP3, CMH-1), caspase-6 (Mch2), caspase-8 (MACH, FLICE, Mch5), caspase-10 (Mch4), caspase-2 (ICH-1), caspase-9 (ICH-LAP6, Mch6) and catalytically active fragments thereof that are relatively inert zymogen fragments.

There has been progress toward understanding cell proteins and particularly enzymes, involved in apoptosis. For example, certain cell proteases such as caspases (i.e. cysteinyl aspartate-specific proteases), *C. elegans* ced-3 and granzyme B have been implicated in apoptosis. Nucleic acid sequences encoding several capsases and proteolytic substrates for same are known. For example, caspase-3 (i.e. CPP32) has been particularly well-studied. See e.g., Thompson, C. B. *Science*, 267:1456 (1995); and Walker, N.P.C. et al. Cell, 78:343 (1994).

In particular, activation of Caspase-3 (Casp3) has previously been shown to be a rubicon of apoptosis by cleavage of the inhibitor of caspase-activated DNAse (ICAD) resulting in the activation of CAD and ultimately cell death. See e.g., Salvesen, G. S., et al., Caspases: intracellular signaling by proteolysis.*Cell* **91**:443 (1997); Henkart, P. A., ICE family proteases: mediators of all apoptotic cell death? *Immunity* **4**: 195 (1996); Cohen, G. M., Caspases: the executioners of apoptosis. *J. Biochem.* **326**: 1 (1997); Woo, M. et al., Essential contribution of caspase 3/Casp3 to apoptosis and its associated nuclear changes, *Genes & Dev.* **12**: 806 (1998); Enari, M., et al., A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**: 43 (1998); Liu, X., et al., DFF40 induces DNA fragmentation and chromatin condensation during apoptosis. *Proc. Natl. Acad. Sci. USA* **15**:8461 (1998). In addition, activated Casp3 can catalyze the activation of inactive Casp3, thereby further amplifying the apoptotic signal.

The structure of the Casp3 zymogen is known to include a N' terminal Pro domain, followed by a caspase cleavage recognition site, then the p 17 domain that contains the catalytic Cys residue, a second caspase cleavage site and finally the pl2 domain (see Fig. 9A). The zymogen form of Casp3 remains inactive; however, during apoptotic signaling, it is cleaved by upstream caspases, such as Caspase-8 in T cells, resulting in loss of the Pro domain and an active pl7:pl2 heterotetramer. See Woo, M. et al., (supra).

Example 12 below illustrates a specific inactivation of the HIV viral replication machinery to treat HIV infected cells. The strategy exploits the HIV Protease to kill the infected cell while leaving uninfected cells unharmed. As will be more fully described in Example 12, a modified Caspase 3 protein, TAT-Casp3, was made. This fusion protein transduces into ~100% of infected and uninfected cells. However, due to substitution of endogenous cleavage sites for HIV proteolytic cleavage sites, TAT-Casp3 is only specifically activated by HIV Protease in infected cells, resulting in apoptosis, whereas in uninfected cells it remains in the inactive zymogen form. See Ratner, L. et al., Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313**: 277 (1985). By substitution of proteolytic cleavage sites, this strategy could be applied to other pathogens encoding specific proteases, such as Hepatitis C virus, cytomegalovirus and malaria. See Rice, C. M.,Flaviviridae: The viruses and their replication, in *Fields Virology* (eds Fields, B. N., Knipe, D. M., & Howley, P. M.) 931-960 (Lippincott-Raven Publishers, Philadelphia, 1996); Welch, A. R. et al., Herpesvirus maturational protease, assemblin: identification of its gene, putative active site domain, and cleavage site. *Proc. Natl. Acad. Sci. USA* **88**: 10792 (1991); Francis, SE, et al., Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. *Ann. Rev. Microbiol.* **51**: 97 (1997).

See also Ratner, L. et al. (1985), *supra* for disclosure of the complete nucleotide sequence of the AIDS virus. See also, Wong, J.K (1997) *Science* 278: 1291 and Finzi, D. et al. (1997) *Science* 278: 1295; for disclosure relating to treatment of HIV infections.

See the following references for more specific information relating to the structure and function of the HIV virus: Wu, X. et al. *EMBO J.* (1997) 16: 5113; Lillehoj, E.P. et al. (1998); Kohl, N.E. et al. (1988) *PNAS (USA)* 85: 4686; Gottlinger, H.G., et al. (1989), *PNAS (USA)* 86: 5781.

In particular, Example 12 shows production of a transducible, modified apoptotic promoting caspase-3 protein (ie. TAT-Casp3), that

substitutes HIV proteolytic cleavage sites for endogenous sites. Further, the fusion molecule efficiently transduces into ~100% of cells, but remains inactive in uninfected cells. In HIV infected cells, TAT-Casp3 becomes processed into an active form by HIV protease resulting in apoptosis of the infected cell. As will be apparent from the accompanying examples and discussion, this specific strategy is generally applicable and could be used to combat other pathogens encoding specific proteases, such as Hepatitis C virus, cytomegalovirus and malaria.

An additionally preferred zymogen is granzyme B.

An additionally preferred zymogen is Bid. The Bid protein has been reported to be a 20kDa protein related to the Bcl2/Bax family of apoptotic regulatory proteins. See Luo et al. (1998) *Cell* 94: 481; Li et al. (1998) *Cell* 94: 491; Wang et al. (1996) *Genes & Dev.* (1996) 10: 2859. The murine Bid sequence can be found in GenBank, accession number: U75506; NID: g1669513. See Example 11.

Additionally preferred zymogens are, e.g., p53, p19ARF and p14ARF. Both are involved in monitoring/modulating apoptosis in cells. In cancer cells, p53 is highly selected for loss and restoration of p53 to a cell will result in killing of the cancer cell but not the normal cell. Thus this may serve to discriminate between normal and cancer cells. p19ARF is an upstream activator of p53 that under certain circumstances results in the activation of p53 by binding MDM2 an upstream negative regulator of p53. See Kamijo et al. (1998) *Proc. Natl. Acad. Sci USA* 95: 8292; Sherr (1998) *Genes & Dev.* 12:2984.

As noted previously, it has been found that mass action enhances the activity of certain embodiments of the anti-pathogen system. More particularly, it is believed that it is possible to administer the anti-pathogen system in many instances at extremely low doses (i.e., nanomoler levels). This feature can be particularly advantageous as it can enhance cell (and patient) tolerance for the anti-pathogen system.

More specifically, cleavage of the cytotoxic domain appears to draw additional fusion molecules into infected cells, thereby resulting in specific concentration of the cytotoxic domain and the cytotoxin in those infected cells. That concentration can be particularly significant with some cytotoxins, particularly those that require concentration to exhibit optimal effect. Illustrative examples of such cytotoxins include those obtained from zymogens of blood coagulation proteases such as thrombin and fibrin; trypsin, chymotrypsin, diphtheria toxin, ricin, shiga toxin, abrin, cholera toxin, saporin, pseudomonas exotoxin (PE), pokeweed antiviral protein, and gelonin. Additional examples include biologically active fragments of diphtheria toxin A chain and the ricin A chain.

Additionally preferred are cytotoxic domains which include proteins and particularly enzymes such as certain kinases and nucleoside deaminases associated with necrosis. Such enzymes include viral thymidine kinases, e.g., HSV thymidine kinase, and cytosine deaminase, respectively, as well as catalytically active fragments thereof.

Additionally preferred zymogens include those active at the surface of pathogen-infected cells such as a phospholipase enzyme, particularly phospholipase C.

Preferred zymogens and enzymes are generally capable of killing cells as determined by a suitable cell viability assay, e.g., Trypan blue exclusion. More preferred zymogens and enzymes have a molecular weights of between about 5, 10, 20, 30, 40, 50 kD up to about 100 to 500 kD or more as assayed by standard methods. The molecular weight can be determined by a number of conventional techniques such as SDS-PAGE gel electrophoresis, sedimentation centrifugation, and column chromatography.

A particularly preferred zymogen is caspase-3 (CPP32, apopain, Yama) or a catalytically active fragment thereof. See Examples 5-6 below.

Another particularly preferred enzyme is HSV-1 thymidine kinase or a catalytically active fragment thereof. See Example 8 below.

In general, preparation of the fusion molecules of the invention includes conventional recombinant steps involving, e.g., polymerase chain amplification reactions (PCR), preparation of plasmid DNA, cleavage of DNA with restriction enzymes, preparation of oligonucleotides, ligation of DNA, isolation of mRNA, introduction of the DNA into a suitable cell, and culturing of the cell. Additionally, the fusion molecules can be isolated and purified using chaotropic agents and well known electrophoretic, centrifugation and chromatographic methods. See generally, Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd ed. (1989); and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1989) for disclosure relating to these methods.

The following Table I provides examples of pathogen-specific proteases and protease cleavage sites for a number of known pathogens. The listed protease cleavage sites are illustrative of those which can be used in accord with the present invention.

Table I

PATHOGEN	PROTEIN	SEQUENCE
HIV-1:	[1] GAG PROTEINS:	
	p17-p24	SQVSQNY---PIVQNLQ
	p7-p1	CTERQAN---FLGKIWP
	POL PROTEINS	
	P6'-Protease	GTVSFSF---PQITLWQ
	Protease-RT	IGCTLNF---PISPIET
Hepatitis C Virus (HCV):	[2] NS3-NS4A	CMSADLEVVT--STWVLVGGVL
	NS4A-NS4B	YQEFDEMEEC---ASHLPYIEQG

	NS4B-NS5A NS5A-NS5B	WISSECTTPC---SGSWLRDIWD GADTEDVVCC--SMSYTWTGAL
Malaria parasite: Plasmodium falciparum	[3] hemoglobinase	[4] WALERMF---LSFPTTK
1, 2-	sites are listed as between cleaved proteins from larger polyproteins	
3-	Recognizes a specific sequence in alpha hemoglobin.	
4-	cut is between residues 33F-34L	

See also Gluzman, I. Y. et al., *J. Clin. Invest.*, 94:1602 (1994); Grakoui, A. et al., *J. of Virol.*, 67:2832 (1993); Kolykholov, AA. et al., *J. of Virol.*, 68:7525 (1994); and Barrie, K. A. et al., *Virology*, 219:407 (1996), the disclosures of which are incorporated by reference.

Additional pathogen-specific proteases and specified cleavage sites have been described and can be used in accord with the present anti-pathogen system.

For example, an HSV-1 maturational protease and protease cleavage site has been described. See e.g. Hall, M.R.T. and W. Gibson, *Virology*, 227:160 (1997); the disclosure of which is incorporated by reference.

Further, two aspartic proteinases referenced as plasmepsins I and II have been found in the digestive vacuole of P. falciparum. The corresponding proteinase cleavage sites have also been disclosed. See e.g., Moon, R.P., *Eur. J. Biochem.*, 244:552 (1997).

It will be appreciated that any of the above-referenced protease cleavage sites can be modified as desired (e.g., by site-specific mutagenesis) so long as the sites are specifically cleaved by the pathogen-specific protease for which they are intended. In some cases, it may be useful to determine the minimal sequence necessary for specific proteolytic cleavage, e.g., to optimize size and spatial considerations relating to the fusion protein. Such minimal sequences have been reported for many pathogen-specific protease cleavage sites.

Alternatively, the minimal sequence for a desired proteolytic cleavage site can be readily obtained by mutagenesis, particularly deletion analysis and site specific mutagenesis (e.g., alanine scanning mutagenesis). The modified cleavage site can be readily assayed in a standard protease cleavage assay as described below.

By the term "specifically cleaved" is meant that peptide bonds in a specified protease cleavage site are specifically broken (i.e. hydrolyzed) by one or more proteases induced by a pathogen infection. That is, the protease cleavage sites are not broken by proteases which naturally occur in an infected or uninfected cell such as those proteases referred to as housekeeping proteases. Specific cleavage of those protease cleavage sites can be monitored by a variety of techniques including SDS-polyacrylamide gel electrophoretic methods.

Preferred pathogen-specific protease cleavage sites include the HSV-1 protease cleavage sites p17-p24 (SQVSQNY---PIVQNLQ; SEQ ID NO. 9), p7-pl(CTERQN---FLGKIWP; SEQ ID NO. 10), and pr-RT (IGCTLNF---PISPIET; SEQ ID NO. 11). See Table I above and the Examples below.

Particularly preferred fusion proteins include operatively linked in sequence (N to C terminus): 1) TAT or a suitable transducing fragment thereof such as the minimal TAT sequence/ the p17-p24 or protease-RT cleavage site/ HSV TK; 2) TAT or a suitable transducing fragment thereof such as the minimal TAT sequence/ the protease-RT cleavage site/ the large domain of CPP32/ the p17-24 cleavage site/ and the small subunit of CPP32; 3) TAT or a suitable transducing fragment thereof such as the minimal TAT sequence/ the p17-p24 or protease-RT cleavage site/ and p16 wild-type or mutant form thereof and 4) TAT or a suitable transducing fragment thereof such as the minimal TAT sequence/(p7-p1) protease change site/HIV protease.

As mentioned above, it has been found that use of the present anti-pathogen system is facilitated by providing the fusion proteins in a misfolded form. For example, it has been found that native fusion proteins, when used in accord with the present anti-pathogen system, transduce much less efficiently than corresponding misfolded sequences. Thus, it is generally preferred that present fusion proteins be fully or partially denatured prior to use in the present anti-pathogen system. Methods for fully or partially denaturing proteins are well known and include treatment with recognized chaotropic agents such as urea, particularly about 6-8M urea, β-mercaptoethanol, DTT, SDS or other detergents, particularly ionic detergents. Further contemplated are physical treatments capable of denaturing proteins and polypeptides such as heating or sonication. Also envisioned are methods including one or more chaotropic agents and physical treatments.

In preferred methods of the invention, the fusion protein is introduced into the cell as a misfolded fusion protein. As mentioned previously, it has been found that the rate and quantity of fusion protein uptake into the cell is significantly enhanced when compared to the same fusion protein introduced into the same cells in a low energy and essentially native conformation.

The invention further provides nucleic acid sequences and particularly DNA sequences that encode the present fusion proteins. Preferably, the DNA sequence is carried by a vector suited for extrachromosomal replication such as a phage, virus, plasmid, phagemid, cosmid, YAC, or episome. In particular, a DNA vector that encodes a desired fusion protein can be used to facilitate preparative methods described herein and to obtain significant quantities of the fusion protein. The DNA sequence can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include mammalian cell systems

infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. See generally Sambrook et al., *supra* and Ausubel et al. *supra*.

In general, a preferred DNA vector according to the invention comprises a nucleotide sequence linked by phosphodiester bonds comprising, in a 5' to 3' direction a first cloning site for introduction of a first nucleotide sequence encoding a protein transduction domain, operatively linked to a sequence encoding a cytotoxic domain. Preferably, the encoded cytotoxic domain includes additional cloning sites for an encoded potentially toxic molecule such as a zymogen. It is further preferred that the cytotoxic domain include additional cloning sites for encoded protease cleavage sites.

Figures 3A-C depict particularly preferred DNA vectors of the invention. The DNA vectors are derived from the pTAT/pTAT-HA vector illustrated in Fig. 1. Preferred nucleic acid linker sequences for use with the pTAT/pTAT-HA vector are shown in Fig. 2.

In most instances, it will be preferred that each of the fusion protein components encoded by the DNA vector be provided in a "cassette" format. By the term "cassette" is meant that each component can be readily substituted for another component by standard recombinant methods. In particular, a DNA vector configured in a cassette format is particularly desirable when the encoded fusion protein is to be used against pathogens that may have or have capacity to develop serotypes.

More specifically, it is envisioned that in some cases, certain pathogen serotypes may be associated with individual protease cleavage

sites specific for that serotype. In this case, one or more existing protease cleavage sites in a DNA vector formatted as a cassette can be replaced with other pre-determined protease cleavage sites as needed. Particular protease cleavage sites can be selected in accord with presence of the pathogen in individual patients.

More generally, DNA vectors according to the invention formatted as a cassette minimize or eliminate occurrence of pathogen serotypes during treatment of a mammal and particularly a human by providing means to add or replace fusion protein components as needed.

In particular, with respect to pathogenic viruses such as HIV, the DNA vectors are specifically formatted to adapt to specific strains of the virus and future mutation of the virus by providing means to substitute new HIV proteolytic cleavage sites into the fusion protein. These sites can be readily determined in a patient by polymerase chain reaction (PCR) amplification of the DNA obtained from patient and DNA sequencing across the viral cleavage sites using standard oligonucleotide primers. For example, a variety of suitable oligonucleotide primers could be selected for the amplification in accord with published sequences. The new/altered cleavage site can then be inserted into a fusion protein, e.g., the pTAT-CPP32 bacterial expression vector described in the examples below, protein purified and misfolded and then administered to the patient in a relatively short time frame (about 3-4 weeks).

Significantly, the present anti-pathogen system can thus serve as an effective "warning system" that can register changes in pathogen serotype *in vitro* or *in vivo*. In particular, development of pathogen serotypes will be evidenced by decreased killing or injuring by the anti-pathogen system. The ability to rapidly detect appearance of the genetically altered pathogen serotypes is particularly relevant to developing rational therapies and can be remedied, e.g., by modifying the fusion protein as described above and/or by implementing a "cocktail" therapy approach as described below.

The term "cloning site" is intended to encompass at least one restriction endonuclease site. Typically, multiple different restriction endonuclease sites (e.g., a polylinker) are contained within the nucleic acid. It optimal positioning of cloning sites in a DNA vector facilitate the cassette format.

The fusion proteins of the present invention can be separated and purified by appropriate combination of known techniques. These methods include, for example, methods utilizing solubility such as salt precipitation and solvent precipitation, methods utilizing the difference in molecular weight such as dialysis, ultra-filtration, gel-filtration, and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electrical charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatograph, methods utilizing a difference in hydrophobicity such as reverse-phase high performance liquid chromatograph and methods utilizing a difference in isoelectric point, such as isoelectric focusing electrophoresis, metal affinity columns such as Ni-NTA. See generally Sambrook et al. and Ausubel et al. *supra* for disclosure relating to these methods.

It is preferred that the fusion proteins of the present invention be substantially pure. That is, the fusion proteins have been isolated from cell substituents that naturally accompany it so that the fusion proteins are present preferably in at least 80% or 90% to 95% homogeneity (w/w). Fusion proteins having at least 98 to 99% homogeneity (w/w) are most preferred for many pharmaceutical, clinical and research applications. Once substantially purified the fusion protein should be substantially free of contaminants for therapeutic applications. Once purified partially or to substantial purity, the soluble fusion proteins can be used therapeutically, or in performing *in vitro* or *in vivo* assays as disclosed herein. Substantial purity can be determined by a variety of standard techniques such as chromatography and gel electrophoresis.

As discussed above, fusion proteins of the invention can be expressed in insoluble forms. That can avoid proteolytic degradation of the fusion protein, significantly increase protein yields and increase delivery of fusion protein into target cells. The insoluble protein can be purified by known procedures such as affinity chromatography or other methods as detailed above.

As mentioned generally above, a host cell can be used for preparative purposes to propagate nucleic acid encoding a desired fusion protein. Thus a host cell can include a prokaryotic or eukaryotic cell in which production of the fusion protein is specifically intended. Thus host cells specifically include yeast, fly, worm, plant, frog, mammalian cells and organs that are capable of propagating nucleic acid encoding the fusion. Non-limiting examples of mammalian cell lines which can be used include CHO dhfr- cells (Urlaub and Chasm, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)), *293 cells* (Graham et al., *J Gen. Virol.*, 36:59 (1977)) or myeloma cells like SP2 or NSO (Galfre and Milstein, *Meth. Enzymol.*, 73(B):3 (1981)).

Host cells capable of propagating nucleic acid encoding a desired fusion protein encompass non-mammalian eukaryotic cells as well, including insect (e.g., *Sp. frugiperda*), yeast (e.g., *S. cerevisiae, S. pombe, P. pastoris., K. lactis, H. polymorpha*; as generally reviewed by Fleer, R., *Current Opinion in Biotechnology*, 3(5):486496 (1992)), fungal and plant cells. Also contemplated are certain prokaryotes such as E. coli and Bacillus.

Nucleic acid encoding a desired fusion protein can be introduced into a host cell by standard techniques for transfecting cells. The term "transfecting" or "transfection" is intended to encompass all conventional techniques for introducing nucleic acid into host cells, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, viral transduction and/or

integration. Suitable methods for transfecting host cells can be found in Sambrook et al. *supra*, and other laboratory textbooks.

The present invention further provides a production process for isolating a fusion protein of interest. In the process, a host cell (e.g., a yeast, fungus, insect, bacterial or animal cell), into which has been introduced a nucleic acid encoding the protein of the interest operatively linked to a regulatory sequence, is grown at production scale in a culture medium in the presence of the fusion protein to stimulate transcription of the nucleotides sequence encoding the fusion protein of interest. Subsequently, the fusion protein of interest is isolated from harvested host cells or from the culture medium. Standard protein purification techniques can be used to isolate the protein of interest from the medium or from the harvested cells. In particular, the purification techniques can be used to express and purify a desired fusion protein on a large-scale (i.e. in at least milligram quantities) from a variety of implementations including roller bottles, spinner flasks, tissue culture plates, bioreactor, or a fermentor.

More particularly, misfolded fusion protein for use in accordance with the invention can be produced by a variety of methods. For example, in a preferred method, a desired fusion protein is expressed in suitable bacterial cells and then isolated from those cells as inclusion bodies. The fusion protein is subsequently denatured in a strong chaotropic agent such as about 6 to 8 M urea followed by chromatography on a first column to separate the fusion protein from other bacterial cell components which accompany it. The bound fusion protein is then eluted from the column by standard means followed by dialysis in a suitable buffer or additional chromatography on a second column to remove the urea. Such dialysis or chromatography will provide the fusion protein in a mixture of conformations, with only a minor portion in a lowest energy correctly refolded conformation, e.g. about 25 percent of the protein may be in the low energy folded state. As referred to herein, a fusion protein that is at least partially denatured

means that at least a portion of the protein sample, e.g. at least about 10, 15, 20, 30, 40, 50, 60, 70 or 75 percent of the total number of amino acid residues in a substantially pure fusion protein sample, is in a conformation other than lowest energy refolded conformation.

A fusion protein misfolded into a mixture of conformations can then be transduced into desired cells. For example, the fusion protein can be directly added to cultured cells or to media in which those cells are being propagated.

While not wishing to be bound by theory, it is believed that the higher energy denatured forms of a fusion protein of the invention are able to adopt lower energy conformations that can be more easily transduced into a cell of interest. In contrast, the protein in its favored folded conformation will necessarily exist in a low energy state, and will be unable to adopt the relatively higher energy and hence unstable conformations that will be more easily introduced into a cell.

As mentioned previously, the invention thus provides methods of treatment against pathogen infections such as virus infections and diseases associated with viruses, which methods in general will comprise administration of a therapeutically effective amount of one or more of the fusion proteins discussed above to a mammal, particularly a human, suffering from or susceptible to the pathogen infection.

For example, the fusion proteins of the invention be useful to treat cells infected with a virus capable of causing an immunodeficiency disease, particularly in a human. The fusion proteins will be particularly useful to treat retroviral infection in cells and in a human, particularly HIV infected human cells. Specific examples of retroviral infections which may be treated in accordance with the invention include human retroviral infections such as HIV-1, HIV-2, and Human T-cell Lymphotropic Virus (HTLV) e.g. HTLV-I or HTLV-II infections.

The invention also provides methods of treatment of other diseases caused by or otherwise associated with a virus such as influenza including influenza A and B as well as diseases associated with viruses of the herpes family, e.g., herpes simplex viruses (HSV) including herpes simplex 1 and 2 viruses (HSV 1, HSV 2), varicella zoster virus (VZV; shingles), human herpes virus 6, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and other herpes virus infections such as feline herpes virus infections, and diseases associated with hepatitis viruses including hepatitis C viruses (HCV). Examples of clinical conditions which are caused by such viruses include herpetic keratitis, herpetic encephalitis, cold sores and genital infections (caused by herpes simplex), chicken pox and shingles (caused by varicella zoster) and CMV-pneumonia and retinitis, particularly in immunocompromised patients including renal and bone marrow transplant patients and patients with Acquired Immune Deficiency Syndrome (AIDS). Epstein-Barr virus can cause infectious mononucleosis, and is also suggested as the causative agent of nasopharyngeal cancer, immunoblastic lymphoma and Burkitt's lymphoma.

It is contemplated that the pathogen may be present in a virulent, latent, or attenuated form. Also contemplated is a population of pathogens including a mixture of those forms. Examples of particular pathogens of interest are viruses, e.g., CMV, HSV-1, HCV, particularly HCV type-C, HIV-1, HIV-2, KSH, yellow fever virus, certain flaviviruses and rhinoviruses. In addition, the pathogen can be any one of those capable of causing malaria or a medical condition relating to same such as *P. falciparum, P. vivax, P. ovale*, or *P. malariae*. Typically, the plasmodia cause malaria or various medical complications relating to malaria. The invention can be used to treat an existing condition or it can be used prophylactically to prevent infection by one or more pathogens.

The anti-pathogen system and especially the fusion proteins of the invention can be administered to cells *in vivo* or *in vitro* by one or a combination of strategies.

For example, as mentioned above, the fusion proteins can be administered to primary or immortalized cells growing in culture *in vitro* by conventional cell culture techniques that generally include contacting the cells with the fusion protein and allowing the fusion protein to transduce through the cells for a specified period of time. Typically, cell media will be removed from the cells prior to the contact to increase fusion protein concentration.

In addition, the fusion proteins can be administered to cells *in vivo*, for example, by using a specified delivery mechanism suitable for introduction of fusion proteins into those cells. In general, the type of delivery mechanism selected will be guided by several considerations including the location of the cells, the degree of transduction needed to kill or injure cells infected by the pathogen, and the general health of the cells.

In particular, the fusion proteins of the invention may be administered to a normal, particularly a promoter such as a human, a variety of suitable routes including oral, topical (including transdermal, buccal or sublingual), nasal and parenteral (including intraperitoneal, subcutaneous, *intra*venous, intradermal or intramuscular injection. See generally *Reminington's Pharmaceutical Sciences*, Mack Pub. Co., Easton, PA, 1980. Nasal or oral routes leading significant contact believe one or more of the fusion proteins and with airway epithelia, lung tissue being generally preferred.

The fusion proteins of the present invention can be administered as a sole active agent, in combination with one or more other fusion proteins as provided herein or in combination with other medicaments such as reverse transcriptase inhibitors such as a dideoxynucleoside

including AZT, ddI, ddC, d4T, 3TC, FTC, DAPD, 1592U89 or CS92; TAT antagonists such as Ro 3-3335 and Ro 24-7429; and other agents such as 9-(2-hydroxyethoxymethyl) guanine (acyclovir), ganciclovir or penciclovir, interferon, e.g., alpha-interon or interleukin II, or in conjunction with other immune modulation agents including bone marrow or lymphocyte transplants or other medications such as levamisol or thymosin which would increase lymphocyte numbers and/or function as is appropriate.

Additional medicaments that can be co-administered with one or more fusion proteins of the invention include standard anti-malarial such as those disclosed in Goodman, .G. et al. (1993), *The Pharmacological Basis of Therapeutics*, 8th ed. McGraw-Hill Inc. pp. 978-998. Preferred anti-malarial drugs include chloroquine, chloroguanidine, pyrimethamine, mefloquine, primaquaine and quinine.

Administration of two or more of the above-referenced agents including the fusion proteins of the invention is illustrative of a "cocktail" or "cocktail" therapy.

While one or more fusion proteins of the invention may be administered alone, they also may be present as part of a pharmaceutical composition in mixture with conventional excipient, preferably a pharmaceutically acceptable organic or inorganic carrier substances that is generally suitable for oral or nasal delivery as mentioned previously. However, in some cases, other modes of administration may be indicated in which case the fusion protein can be combined with a vehicle suitable for parenteral, oral or other desired administration and which do not deleteriously react with the fusion proteins and are not deleterious to the recipient thereof. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose,

polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously react with the fusion proteins.

For parenteral application, particularly suitable are solutions, preferably oily or aqueous solutions as well as suspensions, emulsions, or implants, including suppositories. Ampules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees or capsules having talc and/or carbohydrate carrier binder or the like, the carrier preferably being lactose and/or corn starch and/or potato starch. A syrup, elixir or the like can be used wherein a sweetened vehicle is employed. Sustained release compositions can be formulated including those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc.

Therapeutic fusion proteins of the invention also may be incorporated into liposomes. The incorporation can be carried out according to known liposome preparation procedures, e.g. sonication and extrusion.

It will be appreciated that the actual preferred amounts of active fusion proteins used in a given therapy will vary according to the specific fusion protein being utilized, the particular anti-pathogen system formulated, the mode of application, the particular site of administration, etc. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art using conventional dosage determination tests conducted with regard to the foregoing guidelines.

In general, for treatment of a pathogen infection, e.g., a viral infections such as an HIV infection, a suitable effective dose of one or more fusion proteins will be in the range of from 0.01 to 100 milligrams per kilogram of bodyweight of recipient per day, preferably in the range of from 0.1 to 50 milligrams per kilogram bodyweight of recipient per day, more preferably in the range of 1 to 20 milligrams per kilogram bodyweight of recipient per day. The desired dose is suitably administered once daily, or several sub-doses, e.g. 2 to 5 sub-doses, are administered at appropriate intervals through the day, or other appropriate schedule.

As noted previously, a preferred mode of administration is in an aerosol format and particularly by nasal or oral routes.

Another aspect of the invention pertains to kits which include the components of the anti-pathogen system of the invention. Such a kit can be used to kill or injure cells infected by one or more pre-determined pathogens. In one embodiment, the kit includes a carrier means having in close confinement therein at least two container means: a first container means which contains one or more fusion proteins of the invention, and an optional second container means which contains a recombinant vector that encodes the fusion proteins.

To use the anti-pathogen system provided using components of the kit, the fusion protein is administered to cells, *in vitro* or *in vivo* in accordance with methods described above.

The invention is widely applicable to a variety of situations where it is desirable to kill or injure cells infected by one or a combination of pathogens. In addition to specified uses described above, the invention is also applicable to studying mechanisms of pathogen infection of eukaryotic cells such as those cells of plant, insect, or animal origin, e.g., as in cells from primates and other mammals such as domesticated

animals including certain birds, dogs, cats, horses, sheep, cows and the like. Additionally, the present invention can be used for protection of crops or foodstuffs against pathogen attack.

In another aspect of the present invention, the anti-pathogen system can be used to screen candidate compounds for therapeutic capacity to inhibit certain proteins and particularly pathogen-specific proteases in infected cells. In particular, a preferred screening method includes transducing the anti-pathogen into desired cells, preferably cultured cells including immortalized or primary cells; infecting the cells with a pathogen, adding a candidate compound with potential therapeutic capacity to inhibit a pathogen-specific protease, and testing the cells for resistance to the pathogen, e.g., by performing a conventional cell viability assay.

The assay is usually compared to a baseline control to determine the effect of the compound of interest on the cell, e.g.. the resulting phenotype. In addition, the candidate compound can be added before, during or after transducing the cells with the anti-pathogen system.

The baseline control may be the cell before introduction of the fusion protein, the cell in which the fusion protein has not been introduced, or the cell in which the fusion protein is non-functional, e.g.. has a non-functional transcription activator region. One or more pre-determined pathogens can be added to the cell either before, after or during administration of the compound.

The candidate compound of interest can be one of several molecules, including cytokines, tumor suppressors, antibodies, receptors, muteins, fragments or portions of such proteins, and active RNA molecules, e.g., an antisense RNA molecule or ribozyme, or a drug. For example, a combinatorial library of derivatives of a known HIV RT inhibitors such as AZT can be readily tested by the present methods. Preferred compounds according to the invention are capable of reducing

cell killing by at least about 40%, 50%, 60%, 70%, preferably at least about 80%, and more preferably at least about 90% or greater as assayed by standard cell viability tests such as by a Trypan blue exclusion test.

A preferred method of screening a candidate compound for therapeutic capacity to inhibit a pathogen-specific protease comprises:

a) transducing a fusion protein of the invention into a population of cells,

infecting the cells with a pathogen capable of expressing or inducing pathogen-specific protease and expressing the protease,

b) contacting the fusion protein with the pathogen-specific protease sufficient to produce a cytotoxin; and

c) correlating any cytotoxic effects to the therapeutic capacity of the candidate compound to modulate the pathogen-specific protease.

The protein transduction domain can be selected from TAT, Antennapedia homeodomain, HSV VP22; a suitable fragment thereof; or any non-naturally occurring sequences that are capable of transduction. The cytotoxic domain can include a caspase and one or more protease cleavage sites.

DNA and protein sequences described herein can be obtained from a variety of public sources including those specifically mentioned. A preferred source is the National Center for Biotechnology Information (NCBI)- Genetic Sequence Data Bank (Genbank) at the National Library of Medicine, 38A, 8N05, Rockville Pike, Bethesda, MD 20894. Genbank is also available on the internet at http://www.ncbi.nlm.nih.gov. See generally Benson, D.A. et al., *Nucl. Acids. Res.*, 25:1 (1997) for a description of Genbank.

Other reagents used in the examples such as antibodies, cells and viruses can be obtained from recognized commercial or public sources such as *Linscott's Directory* (40 Glen Drive, Mill Valley California 94941),

and the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, Md 20852.

All documents mentioned herein are incorporated herein by reference.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

Example 1- Production of a TAT Fusion Protein Expression Vector

A preferred plasmid for TAT fusion protein expression was prepared as follows. A map of that plasmid is depicted in Figure 1 of the drawings. Figure 2 shows a nucleotide sequence (SEQ ID NO:12) and amino acid sequence (SEQ ID NO:13) of the pTAT linker as well as a nucleotide sequence (SEQ ID NO:14) and amino acid sequence (SEQ ID NO:15) of the pTAT-HA linker.

pTAT and pTAT-HA (tag) bacterial expression vectors were generated by inserting an oligonucleotide corresponding to the 11 amino acid TAT domain flanked by glycine residues to allow for free-bound rotation of the TAT domain (G-RKKRRQRRR-G) (SEQ ID NO:16) into the BamHi site of pREST-A (Invitrogen). A polylinker was added C' terminal to the TAT domain (see Figure 1) by inserting a second oligonucleotide into the NcoI site (5' or N') and Eco RI site that contained NcoI-KpnI-AgeI-XhoI-SphI-EcoRI cloning sites. This is followed by the remaining original polylinker of the pREST-A plasmid that includes BstBI-Hind III sites.

The pTAT-HA plasmid was made by inserting an oligonucleotide encoding the HA tag (YPYDVPDYA; SEQ ID NO:17; see Figure 2) where sequence is bold) flanked by glycines into the NcoI site of pTAT. The 5' or N' NcoI site was inactivated leaving only the 3' or C' to the HA tag followed by the above polylinker. The HA tag allows the detection of the

fusion protein by immunoblot, immunoprecipitation or immunohistostaining by using 12CA5 anti-HA antibodies.

The nucleotide and amino acid sequences of each linker are set forth in Figure 2. The pRSET-A backbone encodes ampicillin resistance, fl, ori, ColE1 ori (plasmid replication) and the transcript is driven by a T7 RNA polymerase promoter.

Example 2- Preparation of Misfolded TAT Fusion Proteins

The TAT fusion proteins described below were purified from host cells and purposefully misfolded to enhance transduction. More specifically, the fusion proteins were purified by sonication of transfected BL21(DE3) pLysS cells (Novagen) obtained from a 5 hr 1 L culture. That culture was inoculated with 100ml from an overnight culture in 10 ml of buffer A (8M urea/20mM HEPES (pH 7.2 (100 mM NaCl)). Cell lysates were resolved by centrifugation, loaded onto an Ni-NTA column (Qiagen) in buffer A plus 20mM imidazole. The column was then washed in 10X column volume, eluted by increasing imidazole concentration in buffer A (stepwise) and then applied to a Mono-Q column on an FPLC (Pharmacia) in 4 M urea/20mM HEPES (pH 7.2, (50 mM NaCl)). TAT fusion protein were eluted with a 1 M NaCl step, desalted on a PD-10 desalting column (Pharmacia) into PBS or 20 mM HEPES [pH 7.2]/137 mM NaCl and frozen in 10% glycerol at –80°C.

FITC-labeled TAT fusion proteins were generated by fluorescein labeling (Pierce), followed by gel purification in PBS on an S-12 column attached to an FPLC (Pharmacia) and added directly to culture media.

Example 3- Production of TAT p16 Fusion Proteins

TAT p16 fusion proteins including HIV protease cleavage sites (p17-p24 or p7-p1) were made according to the following method.

A. <u>Preparation of pTAT-(HIV cleavage sites) constructs</u>:

This plasmid was made by inserting double stranded oligomers encoding the p17-p14 and p7-p1 HIV cleavage sites into the NcoI site of pTAT and pTAT-HA. The cleavage consist 14 amino acids, 7 on each side of the HIV cleavage site.

p17-p24 site (57mer), positive strand:
5' CAT GTC AGG CTC CCA GGT GTC ACA GAA CTA TCC AAT CGT GCA GAA CCT GCA GGG CGC 3'
(SEQ ID NO. 18)

p7-p1 HIV cleavage site (60 mer), positive strand:
5' CAT GCA TTC AGG CTG CAC CGA ACG CCA GGC TAA CTT CCT GGG CAA AAT CTG GCC AGG CGC 3'
(SEQ ID NO. 19)

An oligonucleotide corresponding to the HIV cleavage site p17-p24 (SEQ ID No.18) or p7-p1 (SEQ ID NO. 19) was fused to the pTAT vector described in Example 1 (3' to the PTD sites) to produce a pTAT-HIV_1 or pTAT-HIV_2 vector, respectively. The pTAT-$HIV_{1,2}$ vectors served as a parental vectors for the constructs shown for example in Figs. 3A-C. A p16 protein cDNA sequence was fused to the p17-p24 HIV cleavage site to produce an in-frame TAT-p16 fusion protein cDNA (Fig. 3C). A second p16 fusion protein was made by fusing the p16 cDNA to the pTAT-HIV_2 vector. The order of components in each vector construct (N' terminus to C'-terminus) was: HIS-TAT-PTD-CLEAVAGE SITE-p16-PROTEIN, whereby "cleavage site" denotes the p17-p24 or p7-p1 cleavage sites, respectively. The fusion proteins were each purified and misfolded according to the method described in Example 2 above. The TAT-p16 cDNA vectors were propagated in DH5-α bacteria.

Purified p16 fusion proteins were individually transduced into Jurkat T-cells infected by HIV. Methods for infecting the Jurkat T-cells with HIV and transducing fusion proteins are described in examples, which follow. After 4, 8 and 12 hours, the cells are analyzed for cleavage of the fusion protein by

Western/immunoblot analysis using a commercially available anti-P16 antibody (Santa Cruz). As a control, the p16 cDNA was fused to the pTAT vector described in Example 1 to produce the vector shown in Fig. 3D (no HIV protease cleavage site). That vector encoded a p16 fusion protein fused to TAT that was not cleaved in the infected cells. However, efficient cleavage was observed with p16 fusion proteins encoded by vectors shown in Fig. 3C Tht contained the HIV cleavage sites.

Example 4- Detection Of The p16 Fusion Protein Inside Transduced Cells

To see if p16 fusion proteins could remain in the cell cytosol of the HIV infected cells, the TAT-$HIV_{1,2}$ p16 fusion proteins produced in Example 3 were purified and labeled with FITC as described above. About 35 to 45 nanomoler of the labeled fusion protein was then added to HIV-infected (NLHX strain) and uninfected Jurkat T-cells in accordance with Example 6 below. The transduced Jurkat T-cells were then analyzed by FACS. All (100%) of cells in a mixed population of HIV infected/uninfected cells were transduced with the p16 protein at 1, 2, 4, 8 and 16 hours post-addition of the FITC conjugated fusion protein. However, when the cells were washed in fresh media and the PTD has driven out of the cells due to the concentration gradient (now high inside and 0 outside), the infected cells retained the cleaved substrate (the p16 portion) but uninfected controls lost all of the transduced protein (it transduced out) as determined by the continued presence of FITC-labeled p16 as analyzed by FACS.

Example 5- Production of TAT-CPP32 Fusion Protein

A human CPP32 cDNA (Alnemri et al., *J. Biol. Chem.*, 269:30761 (1994); Genbank Accession No. U13737) was generated by independently PCRing (i.e. performing a Polymerase Chain Amplification (PCR) step) the CPP32 p17 and p12 domains, then adding these DNA fragments together and PCRing using the outside PCR primers. The protocol is outlined in Figure 5. This is called a double PCR cloning approach and is a common

methodological approach to link to two independent DNA fragments together, as follows:

The p17 domain of CPP32 cDNA was PCRed using the A (+ strand) and B(- strand) primers (see below) and the p12 domain by using B(+ strand) and C(- strand) primers. The A primer encodes the p17-p1 HIV cleavage site in-frame with the CPP32 p17 domain coding sequence and the B primer encodes the p17-p24 HIV cleavage site. After this first PCR, the two purified fragments were mixed together and PCRed using only the A(+ strand) and C(- strand) primers. The two DNA fragments base pair together because the 3' end of p17 PCRed domain contains the (-) strand of the 5' end of the p12 PCRed domain. After this PCR, the resultant DNA fragment was digested with XhoI at the 5' end and EcoRI at the 3' end yielding an approximately 900 bp fragment. This fragment was cloned into the XhoI and EcoRI Sites of pTAT and pTAT-HA plasmids. The protein, TAT-CPP32 wild type, was produced in BL2I(DE3) cells as outlined above.

A (+ strand) primer, (90mer):
5' CGC CTC GAG GGC GGC TGC ACC GAA CGC CAG GCT AAC TTC CTG GGC AAA ATC TGG CCA GGC GGA ATA TCC CTG GAC AAC AGT TAT AAA ATG 3'
(SEQ ID NO. 20)

B (+ strand) primer, (72mer):
5' GGC GGC TCC CAG GTG TCA CAG AAC TAT CCA ATC GTG CAG AAC CTG
CAG GGC GGT GTT GAT GAT GAC ATG GCG 3'
(SEQ ID NO. 21)

B (- strand) primer, (75mer):
5' ACC GCC CTG CAG GTT CTG CAC GAT TGG ATA GTT CTG TGA CAC CTG
GGA GCC GCC TGT CTC AAT GCC ACA GTC CAG 3'

(SEQ ID NO. 22)

C (- strand) primer, (37mer):
5' CGA GCT ACG CGA ATT CTT AGT GAT AAA AAT AGA GTT C 3'
(SEQ ID NO. 23)

The order of components in the resulting construct (N' terminus to C'-terminus) was: HIS-TAT-PTD- HIV_2 - subunit of CPP32-HIV_1 - small subunit of CPP32. The vectors encoding the HIS-TAT-PTD- HIV_2 - large subunit of CPP32-HIV-small subunit of CPP32 cDNA fusion proteins were each propagated in DH5-α bacteria. The fusion proteins were expressed and purified as described in Example 2 above. The HIS-TAT-PTD-HIV_2 - large subunit of CPP32-HIV_1 small subunit of CPP32 fusion protein is referred to as "TAT-CPP32".

Example 6- Specific Cell Killing with the TAT-CPP32 Fusion Protein

To show that the TAT-CPP32 fusion protein produced in Example 5 was capable of killing HIV-infected cells, the fusion protein was purified and detectably-labeled with FITC as described above in Example 2. The labeled TAT-CPP32 fusion protein was tested by the following method.

About 5 X 10^6 Jurkat T-cells were infected by HIV (strain NLHX; about 1 x 10^5 to 1 x 10^6 infectious virus per ml). The cells were propagated in RPMI media. Approximately 4 to 7 days after the infection, the media was removed from the plates and about 35 to 45 nanomoler of the TAT-CPP32 fusion protein was added to the cells. The cells were incubated with the fusion proteins for about 30 minutes to allow transduction into the cells. Using FACS analysis, it was found that about 100% of the cells were transduced by the fusion protein. Subsequently, media was added back to the plates and after about 18 hours post-transduction, the cells were examined for cell killing using conventional trypan blue exclusion and microscopy.

It was found that about all of the infected cells were killed by TAT-CPP32. This result is important because it indicates that the TAT-CPP32 fusion protein specifically kills HIV-infected cells but does not kill the uninfected cells in the population.

Example 7- Inhibition of TAT CPP32 Killing Activity

1. Administration of an HIV protease inhibitor

To show that the TAT-CPP32 fusion protein killed HIV-infected Jurkat T-cells by an mechanism that requires specific induction of an HIV-protease, the protease inhibitor Ritonavir (Abbott) was added to infected cells after transduction of the fusion protein. Briefly, cells were infected and transduced as described in Example 6 above. Following the transduction, about 1μg/ml Ritonavir was added to the cell media and allowed to incubate with the cells for about 18 hours. The cells were then assayed for cell killing by conventional trypan blue exclusion and microscopy.

It was found that administration of the Ritonavir essentially blocked the ability of the TAT-CPP32 to kill infected cells. Thus, the TAT-CPP32 fusion protein killing of HIV-infected cells requires an active HIV protease.

2. Mutation of the CPP32 fusion protein (TAT-CPP32 mutant C163M)

To demonstrate that TAT-CPP32 cell killing was due to activation of the CPP32 protein following HIV-specific cleavage, CPP32 was inactivated by mutating the catalytic cysteine at residue 163 to methionine. Briefly, the TAT-CPP32 molecule made in Example 5 was mutagenized to change the catalytically active Cys residue (#163) in the active site to Met by site directed mutagenesis. The following double stranded oligomeric nucleotide was inserted into the StuI site (in the p17 domain at the 5' end of the insert) and PstI site present in the p17-p24 HIV cleavage site between the p17 and p12 domains in TAT-CPP32. The

double stranded oligomer has a blunt end at the StuI 5' end and a 3' overhang at the PstI 3' end.

positive strand oligo (85mer):

5' CC**A TG**C GTG GTA CCG AAC TGG ACT GTG GCA TTG AGA CAG GCG GCT CCC AGG TGT CAC AGA ACT ATC CAA TCG TGC AGA ACC TGC A 3'

(SEQ ID NO. 24)

(bold indicates Cys to Met codon change).

The fusion protein was referred to as "TAT-CPP32^{mut} "or "TAT-CPP32 mutant" to denote the mutated catalytic Cys residue at position 163 of the CPP32 fusion protein.

The TAT-CPP32^{mut} fusion protein was purified and transduced into HIV-infected Jurkat T-cells as described above in Example 6. It was found that the fusion protein was not capable of killing the HIV-infected cells. In contrast, the results of Example 6 show that the TAT-CPP32 fusion protein (with wild-type catalytic Cys residue) specifically killed the HIV infected Jurkat cells.

It is recognized that activation of caspases and particularly CPP32 triggers apoposis and is known in the field as "the point-of-no-return". The results are consistent with this recognition. The results show that the TAT-CPP32 fusion protein specifically kills HIV infected cells perhaps by undergoing apoptosis. In particular, the released CPP32 molecule is believed to start apoptosis only in the infected cells by activating endogenous caspases by cleaving and by cleavage of specific substrates such as PARP.

It is recognized in the field that HIV replication generally requires the presence and specific activity of HIV protease to cleave and process viral polyproteins, such as gag and gag-pol, for maturation as part of its infective life cycle. Transduction of anti-HIV killing molecules into HIV

infected cells undergoing HIV replication, but not uninfected cells, will result in the specific recognition of the engineered HIV cleavage sites in any anti-HIV killing molecule of the invention, converting it from the inactive protein into an active killing molecule. However, uninfected cells do not contain the HIV specific protease and therefore, although present in uninfected cells it will remain in its inactive form.

Without wishing to be bound to any specific theory, it is believed that a cell transduced by the anti-HIV fusion protein such as TAT-CPP32 molecule will undergo apoptosis thereby reducing the viral burst of newly packaged virus particles. In addition, as several proteins are packaged inside the virion, including protease and RT, any escaping packaged virus particles may contain an active anti-HIV killing molecule that could 1) kill the particle prior to infection of a new cell or 2) initiate apoptosis in the newly infected cell, if so it should occur prior to replication of any virus particles.

Example 8- Production And Specific Cell Killing With a TAT-TK Fusion Protein

Figure 4 outlines a method for killing HIV-infected Jurkat T-cells by transducing a fusion protein comprising TK into the cells and then contacting the transduced cells with a prodrug (Acyclovir (Glaxo Wellcome)). TK released from the fusion protein converts the Acyclovir into an active killing molecule, thereby killing the infected cells. However, uninfected (control) cells are not harmed by transduction of the TK fusion protein and administration of the Acyclovir.

The TAT-TK fusion protein was made by the following method. The HSV-1 TK sequence was obtained from Genbank (Accession No. J02224). PCR primers were generated that corresponded to the N' and C' of TK. After PCR, the DNA fragment was cut with NcoI and EcoRI and inserted into the Ncoland EcoRI sites of pTAT-(HIV p17-p24 cleavage site) or pTAT-(HIV p7-p1 cleavage site).

TK forward PCR primer (34MER):
5 'CGG GCC GGC CCC ATG GCT TCG TAC CCC TGC CAT C 3'
(SEQ ID No. 25)

TK reverse PCR primer (39MER):
5' GGC GGG CCG GGA ATT CTC AGT TAG CCT CCC CCA TCT CCC 3'
(SEQ ID NO. 26)

The fusion proteins was each purified and misfolded as discussed above in Example 2.

About 5 X 10^6 Jurkat T-cells were infected by HIV (strain NLHX) as described above in Example 6. Approximately 4 to 7 days after the infection, the media was removed from the plates and about 35 to 45 nanomoler of the TAT-TK fusion protein (p17-p24 or p7-p1 cleavage site) was added to the cells. The cells were incubated with the fusion proteins for about 30 minutes to allow transduction into the cells. Using FACS analysis, it was found that about 100% of the cells were transduced by the fusion protein.

Transduced cells were allowed to incubate for about 18 hours to allow build-up of TK cleaved from the TAT-TK fusion protein. After this time period, the cells were washed in media and allowed to incubate for a further 4 hours. At this point about 1 to 100 nanomoler Acyclovir was added to the plates. After about 3 days, infected and non-infected cells were examined for cell killing by conventional trypan blue exclusion and microscopy. It was found that approximately 100% of the total number of infected cells were killed by administration of the TAT-TK fusion protein and acyclovir.

The results show that the TK enzyme was specifically concentrated in infected cells. However, in uninfected cells, the TK enzyme was not concentrated; the TAT-TK fusion was found to be transduced back out of those cells after washing. Thus, it is believed that the HSV TK processed

the prodrug into an active killing drug only in the cells where it is retained, the infected cells, and not in the normal cells due to the inability of human/mammalian TK to process the prodrug. The results thus demonstrate that the TAT-TK fusion protein is an effective anti-HIV killing molecule.

In addition to TK, an HSV cytosine deaminase cDNA can be readily substituted for the TK gene to provide specific killing or injuring of HSV infected cells in combination with certain nucleoside analogs known in the field.

The TAT-TK and TAT-CPP32 fusion proteins specifically described can be administered to an HIV-infected patient either as an injectable or preferably via an inhalation device to deliver same to the lungs where it will transduce into the blood stream. The fusion proteins will transduce into all contacted cells (airway and lung tissue, blood cells, etc.) including those typically infected by HIV such as certain immune cells in the bloodstream.

Based on experimental and theoretical modeling, it has been reported that average half-life of an HIV infected T cell *in vivo* is approximately 1.6 days. Therefore a preferred treatment protocol for an HIV-infected will be by injection and more preferably inhalation several 7 day periods. Effectiveness of the methods can be monitored by performing well-known manipulations (e.g., PCR) to detect HIV viral particles in biological fluids such as blood. This process is sometimes known as estimating patient viral loads. The manipulations can help determine proper dosing of the fusion protein either alone or in combination with anti-HIV drugs such as those previously mentioned.

Example 9- Transduction of Uninfected Jurkat T-Cells With TAT-HIV Protease

It is recognized that HIV infections can be difficult to monitor *in vitro* and can often vary with respect to the percentage of cells infected.

Additionally, not all cells that can be infected by pathogens are infected by HIV virus. To help overcome these problems and to help further understand induced killing by the TAT-CPP32 fusion protein, co-transduction experiments were performed with an HIV protease fused to TAT. The goal was to co-transduce uninfected Jurkat T-cells with two fusion proteins, the first fusion protein including the cell killing molecule (TAT-CPP32) and the second fusion protein including the HIV protease (TAT- protease) for cleaving the first fusion protein.

Briefly, a transducible HIV protease was constructed by PCR cloning
protease from HIV NLHX strain into the pTAT-(p7-p1 cleavage site) vector. PCR primers were synthesized corresponding to initiating ATG (methionine) of the protease and the translational termination site. The DNA fragment was inserted into pTAT -(p7-pI cleavage site) vector at the NcoI 5'/N' terminal end and the EcoRI site 3'/C' terminal end. The protein was expressed from the plasmid, pTAT-(p7-pl)-Protease, in BL2I (DE3) cells and purified as described above. The fusion protein is referred to as "TAT-Protease."

To test for activation of the TAT-CPP32 by TAT-Protease, 2 x 10^6 Jurkat T cells were placed in 2 ml of culture (RPMI media). To these cells, various combinations of control and experimental transducible proteins were added in 50- 100 nM ranges, as follows:

1. control
2. TAT-Protease (50-100 nM)
3. TAT-CPP32 wild type (50-100 nM)
4. TAT-CPP32 mutant (50-100 nM)
5. TAT-CPP32 wild type plus TAT-Protease
6. TAT-CPP32 mutant plus TAT-Protease
7. Ritonavir (HIV protease inhibitor)
8. TAT-CPP32 wild type plus ritnovir
9. TAT-CPP32 mutant plus ritnovir

The cells were assayed for survival at 18 hr post-addition by trypan-blue exclusionary dye microscopy. TAT-Protease, TAT-CPP32 wild type and mutant proteins showed minimal cytotoxicity when added alone. See Figures 6A and 6B. However, the addition of TAT-CPP32 wild type, but not mutant, plus TAT-protease resulted in a substantial loss of viable cells and hence, activation of the TAT-CPP32 wild type protein. The addition of the protease inhibitor to this experiment resulted in the loss of specific TAT-CPP32 wild type killing. Thus, activation of TAT-CPP32 requires the presence of HIV protease. Taken together, these observations demonstrate the specificity of activation of the TAT-CPP32 protein only in cells expressing HIV protease.

It is believed that the co-transduction method is generally applicable for killing or injuring cells that are not usually infected by HIV virus. Examples of such cells include certain CD4 - (minus) immune cells and non-immune cells such as fibroblasts. Additionally, it will be appreciated that the method is readily adapted to include other transducing fusion proteins described herein, e.g., specified TAT fusion proteins requiring administration of a prodrug (e.g., TAT-TK and Acylcovir).

Example 10- Synthetic Transduction Domains With Enhanced Transduction Efficiency

The following artificial (i.e. synthetic) peptides were made by conventional peptide synthesis as described above. A goal of this experiment was to produce transduction domains that could transduce more effectively as judged by the intracellular concentration in transduced cells. The transduction domains were tested against a suitable control, which typically was the "natural" TAT or an Antp transduction domain. Briefly, a FITC group was synthetically attached to N-terminus of 100% of each peptide so that transduction rate and intracellular concentration of each peptide could be quantified at equilibrium. The TAT transduction domain is recognized to be alpha-helical. In each synthetic peptide sequence, an alpha-helix was modeled with varying amounts of Arg on one face.

That is, a series of synthetic peptides was designed that contained varying amounts of Arg residues on one surface and substituted with Ala residues. Both Ala and Arg residues have the highest probability/energetics for maintaining an alpha-helical structure. See Figure 7, which depicts each peptide as a helical wheel projection. The peptides are shown below in Table 2.

As noted previously, "STD" "SFD" and "PTD" are used herein to specify protein transduction domains

Table 2

SEQ ID NO.	Modified Peptide	Rate Compared to TAT	Intracellular Concentration
1	YGRKKRRQRRR[a]	=	1 X
2.	**A**GRKKRRQRRR	=	1 X
3.	Y**A**RKA**R**RQA**R**R	=	5 X
4.	Y**A**R**AAA**RQ**A**R**A**	=	33 X
5.	Y**A**R**AA**R**AA**RR	=	8 X
6.	Y**A**R**AA**R**AA**R**A**	=	5 X
7.	Y**A**R**RR**R**RR**RR	=	1 X
8.	Y**AAA**R**RR**R**RR**	=	1 X
9.	Y**AAAAAAAAAA**[b]	N.D.	N.D.
10.	RQIKIWFQNRRMKWKK[c]	<	1 X

a. Original TAT domain
b. Insoluble
c. Original ANTP sequence

The synthetic peptides were transduced into Jurkat T-cells along lines described above in Example 4. As can be seen in Table 2, all of the synthetic peptides transduced into the cells. The data show that the synthetic peptides with the most favorable rate and intracellular peptide concentration had the highest probability of having alpha helical structure (compared to naturally-occurring TAT) due to the substituted Ala residues. Further, the best synthetic peptides had Arg residues aligned on a single surface of the helix as suggested by helical wheel diagrams. See Figure 7. In particular, the modified synthetic peptides represented by SEQ ID Nos. 3 to 8 exhibited about a 5 to 10 fold increase in intracellular concentrations when compared to naturally-occurring TAT (SEQ ID NO 1).

The data indicate that it is possible to design synthetic peptides with enhanced transduction efficiency compared with TAT. For example,

the data show that it is possible to increase transduction efficiency of naturally-occurring TAT by increasing probability of alpha helical helix formation in the peptide and by aligning at least two Arg residues on a single peptide helical face. The synthetic peptide sequences shown in Table 2 can be used to increase the transduction efficiency of a variety of fused amino acids, e.g., addition of 2, 5, 10, 20, 50 and 100 amino acids to the synthetic peptide sequence. The synthetic peptide sequences can also be fused to protein sequences of about 10, 15, 20, 30, 50, or about 100, up to about 500 kD or greater. The resulting fusion proteins can be tested for an increase in transduction efficiency as described above.

The naturally-occurring Antp peptide (SEQ ID No. 10) typically exhibits a slower transduction rate than the TAT peptide. Thus, naturally-occurring TAT and the synthetic peptides described above will often be preferred for transducing amino acid sequences and particularly large proteins into cells.

Table 2 shows synthetic peptide sequences that result in the rapid transport by transduction across cellular membranes enhanced into cells. The data show that those peptides having 1) a strong alpha helical nature and 2) at least a face/surface that is covered by Arg. residues are the best transducing domains. Figure 7 below shows a helical wheel plot showing the placement of the residues. All of the synthetic peptides have a transduction rate close to that of TAT (47-57), but some result in an increase intracellular concentration. Particular peptide sequences have at least the face of the helix containing basic residue such as Arg.

Example 11- Killing HIV/Pathogen Infected Cells by Transduction of a Modified TAT-Bid Protein

The pro-apoptotic protein Bid a 20 kDa protein realted to the the Bcl2/Bax family of apoptotic regulatory proteins. Bid is present in a zymogen proform in the cytoplasm. Activation of cells to undergo apoptosis by signaling through receptors such as Fas results in

activation of two separate pro-apoptotic cascades/pathways. Additionally, Caspase-8 activation results in direct cleavage of cytosolic p20 Bid at Asp59 residue (aspartic acid residue #59 in mouse and Asp60 in human). This results in loss of the 5 kDa "pro" domain of Bid and rapid translocation of pl5 Bid to the mitochnodria resulting in release of cytochrome c and mitochondrial poisoning and subsequent death. Thus, Bid exists as an inactive proform/zymogen that can be specifically activated by by proteolytic cleavage resulting in apoptotic induction through a different pathway than Caspase-3 (CPP32) via the DNA degradation pathaway.

A transducible TAT-Bid protein can be made by adding TAT to the N' terminus and removing the endogenous Caspase cleavage site of Bid and replacing it with an HIV cleavage site (TAT-p5 Bid-HIV cleavage-pl5 Bid). The goal was to test the effectiveness of the fusion protein in killing HIV infected cells or cells expressing HIV Protease.

A TAT-HIV clevage-pl 5 Bid protein can also be made to provide a comparison between the two transducible Bid proteins. The cloning strategy is outlined below and, as with the TAT-CPP32 protein, any pathogen protease cleavage site could be cloned into this killing protein. The HIV cleavage site is used in this example as a model system. In addition, killing by TAT-Bid may be more effective than TAT-CPP32 in some cell types/diseases or, more than likely, be complimentary to TAT-CPP32 such that co-transduction of both killing proteins may result in a synergistic effect to further kill the infected cells and potentially at lower concentration levels.

1. Cloning Strategy- Murine Bid was PCR amplified by utilization of the following DNA primers in which the end product results in NcoI (DNA cleavage site) - pS Bid domain - HIV proteolytic clevage site (on the encoded protein) - p 15 Bid domain by performing a double PCR. A TAT-HIV cleavage -plS Bid is also described and under construction.

First, the p5 domain is PCR amplified with primer IF and 2R and in a separate PCR reaction p IS domain is PCRed with primer 2F and 4R. These DNA fragments are purified, mixed together and hybridize via the common regions present in 2F and 2R which are present on the 3' and 5' ends of the respective DNA fragments. The ends of this DNA fragement are extended and a final PCR reaction is performed using only primers 1F and 4R which selects for the full length DNA fragment. This is a common cloning technique. The full length fragment is then cloned/ligated into pTAT-HA by cleavage with NcoI at the 5' end and EcoRI at the 3' end. The resultant plasmid, pTAT-Bid, was transformed into DH5α *E. coli* strain and then into B121(DE3)pLysS *E. coli* strain and protein purified as outlined for the TAT-CPP32 protein. The resultant protein will contact an HIV Protease cleavage site between the TAT-p5 and p15 domains and is designated TAT-p5-HIV-p15 Bid.

TAT-HV-p15 Bid can be constructed similar to the above except only a single PCR reaction is required. The primer 3F contains an NcoI DNA cleavage site followed by the HIV proteolytic cleavage site and DNA sequence homology to the 5' end of p15 Bid domain. The DNA fragment generated from the PCR reaction with with primer 3F and primer 4R is digested with NcoI and EcoRI and cloned into the NcoI and EcoRI sites of pTAT-HA, as outlined above.

2. Primers:

Primer IF (87mer): CgC gCC ATg ggC ggC TCC CAg gTg TCA CAg AAC TAT CCA ATC gTg CAg AAC CTg CAg ggC ggC gAC TCT gAg gTC AgC AAC ggT TCC (SEQ. ID NO. 27)

Primer 2F (52mer): TTC CTg ggC AAA ATC Tgg CCA ggC ggC AgC CAg gCC AgC CgC TCC TTC AAC C (SEQ. ID NO. 28)

Primer 2R (46mer): gTT AgC CTg gCg TTC ggT gCA gCC TgT CTg CAg CTC gTC TTC gAg g (SEQ. ID NO. 29)

Primer 3F (88mer): CgC gCC ATg ggC ggC TgC ACC gAA CgC CAg gCT AAC TTC CTg ggC AAA ATC Tgg CCA ggC ggC AgC CAg gCC AgC CgC TCC TTC AAC C (SEQ. ID NO. 30)

Primer 4R (71 mer): CgC gAA TTC TCA gTC AgC ATA gTC Tgg gAg gTC ATA Tgg ATAgCC gTC CAT CTC gTT TCT AAC CAA gTT CC (SEQ ID NO. 31)

The above-described strategy to clone TAT-p5-HIV-p15 and TAT-HIV-p15 is illustrated in Figures 9A-C.

Example 12- Killing HIV-Infected Cells by Transduction of an HIV Protease-activated Caspase-3 Protein

The HIV Protease-activated Caspase-3 protein was generated to specifically kill cells infected by the HIV virus. The fusion protein was made as follows:

First, a modified Casp3 protein was made by deletion of two residues from the two endogenous caspase cleavage sites (Asp-Ser) on Casp3 and insertion of fourteen residues encompassing the HIV pl7-p24 gag cleavage site ("A" site) and a p7-pl cleavage site ("D" site)20 (Fig. 1A). To introduce the modified Casp3 protein into cells, a previously described method of transducing full length proteins directly into cells was used. See Barrie, K. A., et al., Natural variation in HIV-1 protease, gag p7 and p6, and protease cleavage sites within gag/pol polyproteins: amino acid substitutions in the absence of protease inhibitors in mothers and children infected by human immunodeficiency virus type 1. *Virology* **219**: 407 (1996); Ezhevsky, S. A., et al., Hypo-phosphorylation of the retinoblastoma protein by cyclin D:Cdk4/6 complexes results in active pRb. *Proc. Natl. Acad. Sci. USA* **94**: 10699 (1997); Lissy, N. A., et al., TCR-antigen induced cell death (AID) occurs from a late G_1 phase cell cycle check point. *Immunity* **8**: 57 (1998); Nagahara, H. et al., Highly efficient

transduction of full length TAT fusion proteins directly into mammalian cells: p27 ^{Kip1} mediates cell migration. *Nature Med.* (in press) (1998); Vocero-Akbani, A., et al., Transaction of full length TAT fusion proteins directly into mammalian cells:analysis of TCR-activation induced cell death (AID). In *Methods in Enzymology* (ed Reed, J. C.) (Academic Press, San Diego) (in press) (1998).

Briefly, bacterially produced, misfolded fusion proteins containing an in-frame N' terminal protein transduction domain from HIV TAT are capable of transducing in a rapid and concentration-dependent fashion into ~100% of all target cell types, including: peripheral blood lymphocytes (PBL), all cells present in whole blood, diploid fibroblasts, fibrosarcoma cells, hepatocellular carcinoma cells and leukemic T cells. See Ezhevsky, S. A. et al., Lissy, N. A. et al., Nagahara, H., et al., and Vocero-Akbani, A. et al. (supra). The Pro domain of the modified Casp3 was removed and substituted with the TAT transduction domain resulting in TAT-Casp3WT fusion protein (Fig. 10A). In addition, a catalytically inactive TAT-Casp3 mutant protein was generated by substituting a Met residue for the Casp3 active site Cys'63 residue (TAT-Casp3MUT).

To test the ability of TAT-Casp3 proteins to transduce into cells, TAT-Casp3 proteins were conjugated to fluorescein (FITC), then added directly to the media of Jurkat T cells and analyzed by Flow Cytometry (FACS) (Figs. 10B-C). Both TAT-Casp3WT and TAT-Casp3MUT proteins rapidly transduced into ~ 100% of cells, achieving maximum intracellular concentration in less than 20 min. In addition, based on the narrow peak width before and after addition of FITC labeled proteins, individual cells within the population contain near identical intracellular concentrations of TAT-Casp3-FITC protein. Confocal microscopy analysis showed the presence of TAT-Casp3FITC proteins in both cytoplasmic and nuclear compartments and not merely attached to the cellular membrane. FACS analysis of transduced cells at equilibrium 1 hr post-addition of 3, 6 and 12 nM TAT-Casp3WT-FITC protein demonstrated a

concentration-dependency for protein transduction (Fig. 10E). Thus, TAT-Casp3 proteins readily transduce into 100% of all cells in a rapid and concentration-dependent fashion.

To test the concept of HIV Protease cleavage of transduced heterologous substrates, a model substrate was made by inserting HIV proteolytic cleavage sites into a previously characterized TAT-pl6 fusion protein. See Ezhevsky, S. A. et al. , Lissy, N. A., et al., and Vocero-Akbani, A., et al. (supra). The HIV A cleavage site was inserted between the TAT and pl6 domains, yielding TAT-A-pl6 fusion protein (Fig. 10A). In addition, a transducible HIV Protease (TAT-HIV Pr) was made. See Figure 10A. FITC-labeled TAT-A-pl6, TAT-16 proteins. (See Ezhevsky, S. A., et al., and Vocero-Akbani, A., et al. (supra) and TAT-HIV Pr protein (Fig. 10D) were found to rapidly transduced into 100% of cells.

The generation and transduction of TAT fusion proteins shown in Figures 10A-E are explained in more detail as follows: Figure 10A. Diagram depicting HIV cleavage site sequences and domains of TAT fusion proteins. Figures 10B-D. FACS kinetic analysis of fluorescein (FITC) labeled TAT-Casp3WT, TAT-Casp3MUT and TAT-HIV Pr proteins added to cells at 0, 20 and 30 min. Figure 9E. FACS dose analysis of 3, 6, and 12 nM TAT-Casp3WT-FITC protein added to cells at I hr post-addition. Note rapid, concentration-dependent transduction of all FITC labeled protein into ~ 100% of cells and near identical intracellular concentration within the population as measured by FACS peak width of control vs. transduced cells.

To assay for in vivo cleavage, p 16(-) Jurkat T cells were transduced with 100 nM TAT-Apl6 or control TAT-pl6 protein (no HIV cleavage site) alone or in combination with 50 nM TATHIV Pr fusion protein for 5 hr and analyzed by anti-pl6 immunoblot for in vivo cleavage at the HIV A proteolytic cleavage site (Fig. 11A). Co-transduction of TAT-A-pl6 protein substrate with TAT-HIV Pr resulted in specific substrate cleavage while control TAT-p 16 protein (no HIV cleavage site)

was not cleaved. Size analysis of the cleaved TAT-A-p 16 protein was consistent with retention of the residual HIV half site present on the N' terminus of p 16 (Fig. 11A, see lane 4 vs. 5). It was also noted that the HIV A site was preferentially cleaved over a D site containing TAT-Dpl6 protein in this assay.

Further, the TAT-Casp3MUT protein was transduced in combination with TAT-HIV Pr protein into cells (Fig. 11B). Co-transduction of TAT-Casp3 with TAT-HIV Pr resulted in detection of specific cleavage of TAT-Casp3 at the HIV "A" site between the p 17 and p 12 domains in an HIV Protease-dependent fashion, yielding a TAT-D site-p17-A half site protein. These observations demonstrate that transduced TAT-Ap 16 and TAT-Casp3 proteins containing heterologous HIV cleavage sites can be recognized as substrates by TAT-HIV Protease in vivo.

The in vivo processing in co-transduced cells shown in Figures 11A and 11B is explained in more detail as follows. Figure 11A. Cultures of pl6(-) Jurkat T cells were transduced with TAT-p 16 or TAT-A-p 16 substrate proteins in combination with TATHIV Pr proteins for 5 hr and subjected to anti-p 16 immunoblot analysis. Co-transduction of TATA-pl6 protein with TAT-HIV Pr protein resulted in specific cleavage at the HIV A site. WCE, HepG2 whole cell lysate containing wild type endogenous pl6; A-pl6, cleaved TAT-A-16 product retaining the HIV half site on pl6. Figure 11B. Cultures of Jurkat T cells were transduced with TAT-Casp3MUT protein (TAT-"D" site-p 17 domain-"A" site-pl2 domain) alone or in combination with TAT-HIV Pr (Pr) protein as indicated and immunoblotted with anti-Caspase-3 antibodies specific for the pl7 domain. TAT-D-pl7-A, cleaved product of TAT-Casp3 containing the N' terminal HIV A half site.

In addition, the ability of TAT-Casp3 protein to induce apoptosis in cells co-transduced with TAT-HIV Pr protein was tested. Jurkat T cells were treated with 100 nM TAT-Casp3WT or TATCasp3MUT proteins alone or in combination with 50 nM TAT-HIV Pr protein and assayed for cell

viability 16 hr post-treatment (Fig. 12A). Transduction of TAT-Casp3WT protein alone into cells demonstrated a minor level of cytotoxicity. However, co-transduction of TAT-Casp3WT with TATHIV Pr protein into cells resulted in marked cytotoxicity. Co-transduction of TAT-Casp3MUT with TAT-HIV Pr protein into cells showed only marginal cytotoxicity and also demonstrated an absence of TAT-HIV Protease cytotoxic effects on cells. To further demonstrate the requirement for HIV Protease to activate TAT-Casp3 protein, cells were first pretreated with the HIV Protease inhibitor Ritonavir (1μg/ml), then co-transduced with TAT-Casp3WT or TAT-Casp3MUT in combination with TAT-HIV Pr protein (Fig. 12A). Pretreatment of cells with Ritonavir resulted in protection from the cytotoxic effects of TAT-Casp3WT protein when co-transduced with TAT-HIV Pr protein. Kinetic analysis of TAT-Casp3-dependent cell death demonstrated a linear killing curve with cellular death detected as early as 4 hr post-transduction (Fig. 12B). These results demonstrate that cytotoxicity occurs only in the presence of catalytically active TAT-Casp3WT protein and that activation of TAT-Casp3WT specifically requires active HIV Protease, consistent with HIV Protease cleavage of TAT-Casp3 (Fig. 11B). See Salvensen, G. S., et al., Henkart, P. A., Cohen, G. M., Woo, M., et al., Enari, M. et al., Liu, X., et al. (supra).

The activation of TAT-Casp3 and apoptotic induction in co-transduced cells shown in Figures 12A and 12B is explained in more detail as follows. Figure 12A Cultures of Jurkat T cells were transduced with combinations of TAT-Casp3WT (WT), TAT-Casp3MUT (MUT) and TAT-HIV Protease (Pr) proteins for 16 hr and analyzed for cell viability. Cotransduction of TAT-Casp3WT with TAT-HIV Pr protein resulted in specific cytotoxicity, whereas transduction of TAT-Casp3MUT with TAT-HIV Pr did not. Inclusion of HIV protease inhibitor Ritonavir (Rit) blocked activation TAT-Casp3WT protein and protected cells from cytotoxic effects. Figure 12B Cultures of Jurkat T cells were co-transduced with TAT-Casp3WT (WT), TAT-Casp3MUT (MUT) proteins in combination with TAT-HIV Pr (Pr) protein and analyzed for kinetics of cell viability as indicated.

Additionally, transduced cultures were tested for degraded genomic DNA by TUNEL assay. See Coates, P. J., Molecular methods for the identification of apoptosis in tissues. *J. Histotechnology* **17**: 261 (1994). Transduction of 100 nM TAT-Casp3WT, 100 nM TAT-Casp3MUT or 50 nM TAT-HIV Pr proteins alone into cells showed only background levels of TUNEL positive cells (Fig. 13A). However, co-transduction of TAT-Casp3WT with TAT-HIV Pr protein resulted in a marked increase in TUNEL positive cells. Co-transduction of TAT-Casp3MUT with TAT-HIV Pr protein showed only background TUNEL positive cells (Fig. 13A). Activation of TAT-Casp3 was also assayed by its ability to cleave an artificial Caspase-3 substrate. Jurkat T cells were treated with 100 nM TAT-Casp3WT or TATCasp3MUT proteins alone or in combination with 50 nM TAT-HIV Pr protein for 6 hr and then assayed for cleavage of DEVD-AFC by release of fluorescent AFC (Fig. 13B). See Xiang, J., et al., Bax-induced cell death may not require interleukin 1B-converting enzyme-like proteases. *Proc. Natl. Acad. Sci. USA* **93**: 14550 (1996). Consistent with the TUNEL results from above, co-transduction of TAT-Casp3WT and TAT-HIV Pr proteins into cells resulted in a marked increase in caspase activity that was greater than αFAS treatment.

The HIV Protease activates TAT-Casp3WT protein shown in Figures 13A-B are explained in more detail as follows. Figure 13 A. Cultures of Jurkat T cells were cotransduced with TAT-Casp3WT (WT) and TAT-HIV Pr (Pr) protein resulted in specific TUNEL positive cells, an apoptotic end-marker. However, transduction of TAT-Casp3MUT (MUT) in combination with TAT-HIV Pr protein and singular transduction of TAT-Casp3WT, TAT-Casp3MUT or TAT-HIV Pr proteins remained at background levels of TUNEL positive cells. Abscissa: TUNEL positive cells per high-powered microscopic field; Ctrl, control addition of PBS to cultures; error bars represent SD. Figure 13 B. Transduction of TAT-Casp3WT in combination with TATHIV Protease as above results in specific activation of caspase-3 activity as measured by DEVDAFC

cleavage and AFC fluorescence reported as enzyme activity. Ctrl, control PBS addition; ocFAS crosslinking, positive control.

Transduction of TAT-Casp3WT, TAT-Casp3MUT or TAT-HIV Pr alone showed only background levels of caspase activity. In addition, the appearance of cells containing <2N DNA content was detected in TAT-Casp3 and TAT-HIV Pr co-transduced cells, a classic hallmark of Caspase-3 induced apoptosis as opposed to necrosis. See Woo, M. et al. (supra). These observations demonstrate that transduced TAT-Casp3WT protein remains inactive in cells lacking HIV Protease, but becomes specifically activated in cells harboring active HIV Protease inducing hallmarks of apoptosis and ultimately death.

Due to the absence of a general animal model for HIV, it was of interest to determine if TATCasp3WT protein could kill cells infected with live HIV in culture.

To make this determination, Jurkat T cells were infected for 7-14 days with the NLHX strain of HIV-I and examined microscopically for HIV cytopathic effects. See Westervelt, P., et al., Identification of a determinant within the HIV-1 surface envelope glycoprotein critical for productive infection of cultured primary monocytes. *Proc. Natl. Acad. Sci USA* **88**: 3097 (1991). At the start of each transduction experiment approximately 50% of the culture was HIV positive. HIV infected cultures were transduced for 16 hr with 100 nM TAT-Casp3WT or TATCasp3MUT protein and then assayed for cell viability (Fig. 14). Treatment of HIV infected cells with TAT-Casp3WT protein resulted in a dramatic loss of HIV positive cells from the cultures. In addition, we detected both the appearance of cells containing <2N DNA content and cells with condensed nuclei in TAT-Casp3 treated cells. However, transduction of TATCasp3MUT protein showed negligible effects.

To determine if TAT-Casp3WT induced apoptosis was dependent on active HIV Protease in the infected cultures, HIV infected cultures

were pretreated with 1 μg/ml Ritonavir prior to transduction with 100 nM TAT-Casp3WT protein (Fig. 5). Consistent with the experiments above, pretreatment of HIV infected cultures with Ritonavir resulted in protection from TAT-Casp3WT induced apoptosis. In addition, the observed increased survival of Ritonavir treated cells is consistent with increased longevity of protease inhibitor treated HIV infected cells. See Coffin, J. M., et al. (supra). These observations demonstrate specific killing of HIV infected cells by TAT-Casp3WT protein containing active HIV Protease and the lack of apoptotic induction in cells devoid of HIV protease activity.

The specific killing of HIV infected cells shown in Figure 5 is explained in more detail as follows. Jurkat T cells were infected for 7-14 days with HIV strain NLHX, then transduced with TAT-Casp3WT (WT) or TAT-Casp3MUT (MUT) proteins for 16 hr and assayed for cell viability. TAT-Casp3WT protein efficiently kills a large percentage of HIV positive cells with a single administration, whereas the catalytically inactive TAT-Casp3MUT proteins has no effect. Pretreatment of HIV infected cells with HIV Protease inhibitor Ritonavir (Rit) protects infected cells from TAT-Casp3WT protein killing. Ctrl, control addition of PBS to cultures; abscissa, % viability of HIV positive cells in the population at start of transduction; error bars represent SD.

The present example demonstrates a novel strategy to kill HIV infected cells. Importantly, this strategy harnesses the HIV encoded Protease by utilizing a modified zymogen form of an apoptotic inducer, Casp3, combined with a protein transduction delivery system. The results show that the transduction of proteins into cells is a rapid, concentration-dependent process that targets ~100% of cells. Importantly, TAT-Casp3 protein remains inactive in uninfected cells and is specifically activated by HIV Protease-dependent cleavage in HIV infected cells. This degree of specificity suggests that killing HIV infected cells by such a strategy may result in a high therapeutic index in patients.

As discussed, current treatment of HIV infected cells with protease inhibitors results in increased longevity of infected cells. In direct contrast to treatment with protease inhibitors, TAT-Casp3 protein specifically kills HIV infected cells. In addition, selection for mutations that renders the HIV Protease resistant to a broad spectrum of inhibitors is a continuing and growing problem in combating the HIV epidemical. However, by substituting HIV cleavage sites, the approach provided in this example and elsewhere in this disclosure allows for the continual adaptability of TAT-Casp3 proteins to HIV strain proteolytic cleavage site variance and/or mutation.

The TAT-Casp3 proteins described herein can be used to combat other pathogens by manipulating the proteins to contain relevant pathogen specific protease cleavage sites.

The following methods were used in this example.

1. Cell culture- p16(-) Jurkat T cells (ATCC) were grown as described. See Lissy, N. A., et al. (supra). For in vivo substrate cleavage, 1 X10^6 cells were transduced with 100 nM TAT-p16, TAT-A-p16, TAT-Casp3MUT and/or 50 nM TAT-HIV Pr proteins for 1, 5 or 8 hr as indicated and analyzed by anti-p16 (Santa Cruz) or anti-Casp3 (Pharmingen) immunoblot analysis. See Ezhevsky, S. A., et al. (supra). For cytotoxicity of TAT-Casp3 on uninfected cells, 1 X10^6 cells were transduced with 100 nM TAT-Casp3WT, TAT-Casp3MUT and/or 50 nM TAT-HIV Pr proteins for 16 hr and assayed for viability by Trypan Blue exclusion and/or genomic DNA damage by TUNEL assay (Trevigen). Number of TUNEL positive cells reported as per high-powered microscopic field with four fields per experiment averaged. TAT-Casp3 activity was measured by incubation of 20 μg of whole cell lysate with 50 μM DEVD-AFC and fluorescent AFC formation measured on a FL500 microplate fluorescence reader (Bio-Tek) as described. See Xiang, J., et al., (supra). Cells were preincubated with 1 μg/ml Ritonavir (Abbott

Labs) for 1 hr prior to transduction. For cytotoxicity of TAT-Casp3 on infected cells, Jurkat cultures were infected with 100 ng p24 Ag equivalent NLHX HIV- I strain for 7-14 days, assayed microscopically for cytopathic effects, then replated at 1 x 10^6/ml and transduced with 100 nM of TAT-Casp3WT or TAT-Casp3MUT proteins for 16 hr followed by exclusionary dye viability analysis. Infected cells were pretreated with 1μg/ml Ritonavir for 24 hr prior to transduction with TAT-Casp3WT protein. See Xiang, J., et al., (supra). For Flow Cytometry (FACS) analysis, fluorescein (FITC) conjugated TAT fusion proteins were added to Jurkat T cell culture media and 1 x10^4 cells were analyzed by FACS as described. See Ezhevsky, S. A., et al. (supra).

2. TAT fusion proteins- pTAT-A/D-pl6 expression vectors were constructed by inserting double stranded oligomeric nucleotides encoding 14 residues of the HIV pl7-p24 ("A") cleavage site (single amino acid code: SQVSQNY-PIVQNLQ SEQ ID NO. 9) or the HIV p7-p 1 ("D") cleavage site (CTERQAN-FLGKIWP; SEQ ID NO. 10) into the Nco-I site of pTAT-pl6. See Ratner, L., et al., Welch, A. R., et al., Nagahara, H., et al., and Vocero-Akbani, A., et al. (supra). pTAT-Casp3WT vector was constructed by independent PCR amplification of the pl7 and pl2 domains containing engineered HIV A and D cleavage sites (14 residues) into the primers (pl7-forward primer:
5'-CGCCTCGAGGGCGGCTGCACCGAACGCCAG
GCTAACTTCCTGGGCAAAATCTGGCCAGGCGGAATATCCCTGGACAACAG
TTATAAAATG-3' (SEQ ID NO. 32) ;
pl7-reverse primer: 5' CCGCCCTGCAGGTTCTGC ACGATTGGATAGT
TCTGTGACACCTGGGAGCCGCCTGTCTCAATGCCACAGTCCAG 3' (SEQ ID NO. 33);
p 1 2-forward primer: 5'-GGCGGCTCCCAGGTGTCACAGAA
CTATCCAATCGTGCAGAACC TGCAGGGCGGTGTTGATGATGACATGGCG
3' (SEQ ID NO. 34);
pl2-reverse primer: 5'-CGAGCTACGCG
AATTCTTAGTGATAAAAATAGAGTTC 3'; (SEQ ID NO. 35)

followed by mixing PCR products and PCR amplification using the p l 7-forward and p 1 2-reverse primers (p l 7-reverse and p 1 2-forward primer sequences overlap). The resultant PCR fragment was subcloned into pTAT-HA23 24 resulting in a TAT-D-pl7-A-pl2 configuration (see Fig. 10A). pTAT-Casp3MUT vector was constructed by inserting a double stranded oligomeric nucleotide (positive strand:5'-CCATGCGTGGTACCGA ACTGGACTGTGGCAT TGAGACAGGCGGCTCCCAGGTGTCACAGAACTATCCAATCGT GCAGAACCTGCA-3'; (SEQ ID NO. 36) containing a Met residue for the active site Cys'63 residue into the Stu-I and Pst-I sites of pTAT-Casp3WT vector.

pTAT-HIV Pr vector was constructed by PCR cloning the HIV Protease gene from HXB2R HIV strain (forward primer: 5' CGGTCCATGGGCGGCG GCCCTCAGGTCACTCTTTGGCAACG 3' (SEQ ID NO. 37) ; reverse primer: 5'CGGGAATTCTCAAAAATTTAA AGTGCAACCAATCTG-3' (SEQ ID NO. 38) and cloning into pTAT23 24. Briefly, TAT fusion proteins were purified by sonication of high expressing BL21(DE3)pLysS (Novagen) cells in 8 M urea, purified over a Ni-NTA column and misfolded on a Mono S column as described23 24. FITC conjugated TAT fusion proteins were generated by fluorescein isothiocyanate labeling (Pierce), followed by gel purification in PBS on an S-12 column attached to an FPLC (Pharmacia) or PD-10 desalting column (Pharmacia), then added directly to cells in culture media and analyzed by FACS or microscopy.

Example 13- Production and Testing of Class II Synthetic Transduction Domains

In the course of further investigation of synthetic transduction domains that require a strong alpha helical structure with a face of Arginine residues down the helical cylinder, now referred to as Class I type of synthetic transduction domains, an apparent second class of transduction domains, termed Class II type was discovered. Class II synthetic transduction domains also require basic

residues, such as Arginine or Lysine, but preferably Arginine; however, the introduction of kinks in the secondary structure due to the inclusion of Proline residues distinguishes them from Class I domains.

Below are listed six examples of Class II type of synthetic transduction domains and their relative transduction potential compared to HIV TAT 47-57, using single letter amino acid code:

Table 3

SEQ ID #: to TAT:		Relative Transduction Potential
36	YARAARRPRRR	5x
37	YARAPRRARRR	3x
38	YARAPRRPRRR	3x
39	YARAAARPARA	unknown
40	YARAPARQARA	unknown
41	YARAPARPARA	unknown

Example 14- Targeted Protein Transduction in the Mouse

To ascertain if the PTD could mediate transduction of proteins into tissues and cells of mammals, a series of *in vitro* and *in vivo* experiments were performed.

The protocol is briefly described as follows. Balb6 mice were injected intraperitoneally (IP) with 500 µL of control PBS, Synthetic Protein Transduction Domain #4 (STD-4; ~2,000 Da) or TAT-Cyclin-Dependent Kinase Dominant-Negative protein (TAT-Cdk2 DN; ~39,000 Da). The STD (PTD) sequence is shown above in Table 2. STD-4 and TAT-Cdk2 DN were labeled with a fluorecein (FITC) molecule (~550 Da) to permit analysis of tissues and cells by both Flow Cytometry analysis (FACS), 100,000 cells analyzed per sample, and confocal microscopy where a 488 nM laser excites the fluorecein resulting in green light emission that can be detected by detectors (these are standard pieces of experiment and prototcols). Blood samples were taken from the mice at periodic intervals and analyzed by FACS. At the point where the blood cells reached equilibrium with the transduced FITC labeled peptide/protein, the animal was sacrificed and either a splenectopy performed for FACS analysis and/or tissues of the mice were frozen in O.C.T. media and liquid nitrogen for sectioning on a cryostat for confocal microscopy.

Analysis of blood at 15 min intervals from a mouse injected with TAT-Cdk2 DN-FITC protein resulted in a final total body concentration of 100 pM is represented in Figure 17A. The blood cells, which include ALL cells present in blood (RBCs, PMNCs, T & B cells, etc.), were positive for TAT-Cdk2 DN-FITC signal as early as 15 min post-IP injection. The cells continued to increase in FITC emission until 45 min whereby the signal appeared to level off, though a slight increase was noted at 90 min (Figure 17A). The control blood sample was a bleed prior to IP injection from the same mouse. At 90 min post-IP injection, the mouse was

sacrificed and a splenecotmy performed. The spleen was made into a near single cell suspension by the standard method of rolling it between two frosted slides. The splenocytes were then analyzed by FACS for FITC emission. 100% of the splenocytes were positive for a 10x increase in FITC emission compared to a control mouse. See Figure 17B.

Analysis of 10 min intervals of a mouse IP injected with STD-4-FITC peptide at a final body concentration of 10 nM is presented in Figures 18A-B. FACS analysis of blood at 10 min and 20 min showed that equilibrium of STD-4-FITC in all blood cells was achieved in less than 10 min (Figure 18A). Control was a pre-bleed of the mouse prior to IP injection. Analysis of splenocytes (see above for procedure) 20 min post-injection showed that all cells present in the spleen were transduced (Figure 18B). The control was a PBS injected mouse.

Additional STD-4-FITC IP injected mice were analyzed for transduction into tissues by sacrificing the mouse 25 min post-injection and taking tissue samples, including: brain, muscle, spleen, pancreas, liver, heart and kidneys. The tissues were frozen in O.C.T. media and 10 μm sections fixed to glass slides. Confocal microscopy analysis of brain and muscle tissue compared to these tissues from a PBS injected mouse revealed a strong and universal FITC emission from the STD-4-FITC injected mouse tissues. See Figures 19A-D. Please note that the dark spots on the brain sample are due to freezer burn of the tissue which results in loss of the tissue from that section of the slide. Thus, the entire brain and muscle were transduced with STD-4-FITC consistent with our previous in vitro tissue culture results.

The results show that proteins can be transduced into the brain, muscle, spleen and blood of mammals. In addition, from these observations one can assume that every organ, tissue and cell of the mouse was also transduced. Indeed, the ability to efficiently transduce proteins across the blood: brain barrier, now permits the introduction of 1.) full length proteins and domains to combat diseases such as

Alzheimer's, Huntingtons and Parkinson and as well as 2.) the ability to deliver existing small molecules that could not otherwise be delivered to the brain or other cells in the body due to detrimental properties of these small therapeutic molecules such too large in size (> 600 Da) or biophysical properties such as hydrophobic, etc.

FITC which is 550 Da and has no bioavailability and hence cannot enter or transduce into cells on its own. Thus, by coupling FITC to TAT PTD or STD-4 we have directly demonstrated the ability of TAT PTD and STD-4 to transduce FITC efficiently into 100% of the cells in the brain, blood, muscle and spleen and likely, it was transduced into 100% of all cells in the mouse. These observations directly demonstrate the utility of PTDs and STDs to deliver both large proteins and small compounds into cells that have no bioavailability.

Additional experiments were performed to confirm results of this example.

As a first step, we synthesized a 15-mer peptide containing the 11 amino acid TAT PTD sequence labeled with an N' terminal fluorescein group (FITC)-Gly-Gly-Gly-Gly motif during synthesis. This resulted in identical coupling rates (99%) between peptides (FITC-G-GGG-YGRKKRRQRRR). All peptides were resuspended in water and concentrations normalized by fluorescence values from a Fluorometer. FACS kinetic analysis was then performed on Human Jurkat T cells by injection of 20ul of TAT-FITC-labeled peptide or control FITC with no PTD domain, directly into a sample undergoing FACS analysis. Fluorescent confocal microscopy was performed on peptide treated Jurkat cells fixed in 4% paraformaldehyde.

Kinetic flow cytometry (FACS) analysis of cells treated with the TAT peptide demonstrated a rapid rate of transduction into approximately 100% of cells in less than 30 sec (Fig. 18A-B). In addition, the similar auto-fluorescence peak widths before and after TAT-FITC peptide treatment suggested that all cells had a near identical intracellular

concentration of the peptide. Fluorescent confocal microscopy analysis of TAT peptide treated cells confirmed the presence of the TAT-FITC peptide within the cytoplasm and nucleus of cells, whereas control FITC was merely bound to the cellular membrane (Fig. 19A-D).

Figures 18A-B and 19A-D are explained in more detail as follows. Characterization of TAT-FITC peptide in vitro and in vivo. Flow cytometry analysis of whole blood cells (18A) and splenocytes (18B) from mice 20 min postI.P. injection with TAT-FITC peptide or control free FITC. (D) Fluorescent confocal microscopy of brain (19A,B) and skeletal muscle (19C,D) tissue sections from mice 20 min postI.P. injection with TAT-FITC peptide or control free FITC.

Transduction of TAT PTD peptides into ~100% of all cells assayed *in vitro* was found to be very efficient. Thus it was thought TAT PTD peptides could be delivered into mice via an intraperitoneal (I.P.) injection that would result in both uptake via the lymphatic system (which drains the peritoneal cavity) and transduction across the peritoneum into the blood stream, followed by transport throughout the mouse. To achieve this goal, multiple cohorts of C57BL/6 mice were I.P. injected with 1.7 nmol of TAT-FITC peptide or control free FITC in 500 µl PBS and analyzed for the appearance of fluorescent positive cells at various time intervals.

The protocol employed is more specifically explained as follows: Over forty 4-8 week old C57BL/6 and 129 mice were injected intraperitoneally (I.P.) with 1.7 nmol of TAT-FITC peptide in 500 µl of PBS or 100-500 µg of TAT-β-gal and β-gal control protein present in 0.5-2.0 ml PBS/10% glycerol. Blood was isolated from the orbital artery and splenocytes were isolated by the frosted slide method at indicated time points. Animals were sacrificed tissues harvested and frozen in O.C.T. media. 10-50 µm sections were cut on a cryostat, fixed in 0.25% gluteraldehyde for 15 min and developed as required in 0.2% X-gal solution or analyzed by fluorescent confocal microscopy.

Flow cytometry analysis of whole blood isolated 20 min post-I.P. injection with TAT-FITC peptide demonstrated a strong fluorescent signal in ~100% of blood cells compared to the untreated, pre-bleed control blood cells from the same mouse (Fig. 18A). Consistent with the in vitro peri-membrane binding of control FITC, blood cells from mice injected with control free FITC resulted in a low increase in background fluorescence that was likely due to lymphatic system uptake from the peritoneum. Individual splenocytes were isolated from mice 20 min post-IP injection by performing a splenectomy followed immediately by flow cytometry analysis (Fig. 18B). FACS analysis revealed transduction of TAT-FITC peptide into all splenic cells, including T cells, B cells and macrophages. Control I.P. injections of equal molar amounts of control free FITC showed only a minor increase above background levels of splenocytes. Thus, consistent with the *in vitro* observations, I.P. injection of TAT PTD peptides resulted in a rapid transduction into all cells present in the blood and spleen *in vivo*.

The blood:brain barrier remains a significant boundary that can severely limit the delivery of compounds, peptides and proteins into the brain. Therefore, tissue sections were analyzed from the brain and skeletal muscle of mice 20 min post-I.P. injection with TAT-FITC peptide compared to control free FITC injected mice (Fig. 19A-D). Skeletal muscle and brain tissues were dissected from injected mice, frozen, and cryostat sections prepared. Fluorescent confocal microscopy analysis of 10 μm brain sections revealed a strong fluorescent signal present in all areas of the brain from TAT-FITC peptide injected mice that was significantly above background fluorescence from control mice (Fig. 19A-D). In addition, fluorescent photo bleaching was observed when TAT -FITC peptide sections were subjected to prolonged excitation, further supporting the presence of TAT-FITC peptides in the brain section. Fluorescent confocal analysis of skeletal muscle (quadriceps) also showed significant fluorescent signal compared to controls throughout the sections (Fig. 19C-D). Thus, I.P. injection of TAT PTD peptide resulted in

a rapid transduction into the blood and throughout the mice, and most importantly, across the blood:brain barrier.

As another example to illustrate the efficiency of PTD in directing protein transduction of tissues and cells of mammals, STD4 from Table 2 was fused to FITC and delivered into Balb6 mice. The protocol is outlined generally in Figure 14.

Example 15- Treatment of Circulatory System Diseases Using Transducable Fusion Molecules

It is possible to treat blood vessel diseases and heart diseases by injecting a low concentration of a desired fusion molecule of this invention directly into the blood. In this instance, limited transduction of the fusion molecule will result in cells surrounding the blood vessels. As has been disclosed above and related in the co-pending U.S. Provisional Application Serial No. 60/083,380, transduction in accord with this invention is concentration-dependent and particularly first order. This characteristic of the transduction will facilitate entry of the fusion molecule into the blood and surrounding vessels. A high concentration will drive to total body equilibrium whereas a low concentration will travel through and into a much reduced number of cells.

Methods for selectively perfusing circulatory organs such as the heart and related blood vessels are known in the field and can be used to transform the heart with a fusion molecule of interest.

Example 16- Treatment of Menopause and Related Disorders Using Transducable Fusion Molecules

As discussed, disclosed methods for introducing fusion molecules and particularly fusion proteins into cells are highly efficient and can be targeted to one cell up to an entire mammal as needed.

In this example it would be useful to complement osteoblast function. It is recognized that osteoblasts make bone and osteoclasts damage (adsorb) bone. Here, it should be possible to introduce a protein that binds OSF-2 (transcription factor that drives manufacture of bone in an osteoblast) by the methods disclosed herein. Illustrative proteins can be obtained by performing conventional yeast two-hybrid and related assays (see e.g, literature authored by Brian Seed and others for disclosure relating to conducting these assay). A preferred protein would have a strong transcriptional transactivation domain. Transduction into a subject mammal and particularly a female patient would produce more bone. The expression of OSF-2 is believed to be targeted in specific cell types and specifically those related to bone. Thus, while the transduced fusion protein can be introduced into essentially the entire patient, substantial activity is constrained to those cells that make OSF-2.

Significantly, this method will increase bone formation augmented by the osteoblasts.

Those of skill in this field will appreciate that this concept can be extended to encompass other transcription factors besides OSF-2 as well as medical conditions impacted by those transcription factors.

Example 17- Production and Use of a *"Blue"* Mouse: Protein Transduction In the Mouse Brain and Other Organs, Tissues and Cells.

The foregoing discussion and particularly Example 15 above demonstrates the ability of peptides to rapidly transduce into the mouse. In the present Example it was desired to see if it was possible to transduce full length, biologically active proteins into mammals such as the mouse. A previous attempt to transduce beta-gal chemically cross-linked to the TAT PTD into mice was reported to result in background activity in limited tissues with no activity detected in the kidney or brain. See Fawell et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:664. In contrast to that study, the present Example 17 shows that it is indeed possible to transduce

very large proteins (e.g., 120kDa or more) into the mouse as essentially intact and biologically active molecules.

More particularly, a transducible β-gal protein was generated by inserting the β-gal open reading frame DNA into the pTAT-HA bacterial expression plasmid, producing an N' terminal TAT-β–gal fusion protein (120 kDa) (Fig. 20A). In addition, a control β-gal fusion protein was generated by deletion of only the 11 amino acid TAT PTD while retaining the remainder of the N' terminal leader (119 kDa). TAT-β-gal and control β-gal fusion proteins were purified from bacteria and assayed for the ability to both transduce into cells and retain enzymatic activity in vitro (Fig. 20B). Immunoblot analysis showed the rapid transduction of TAT-B-gal protein into cells, reaching near maximum intracellular concentration in less than 15 min., whereas control β-gal protein failed to transduce into cells even at 2 hr post-addition (Fig. 20B). Unexpectedly, biochemical analysis for β-gal activity of treated cells showed a significant lag between the time to reach intracellular equilibrium and the kinetics of enzymatic activity (Fig. 20C). Transduced proteins become partially denatured during transduction due to the apparently rigorous mechanism of transduction and therefore, this discrepancy likely reflects a slow post-transduction refolding rate of bacterial proteins by human chaperones, such as HSP90. These observations demonstrate the ability of a 120 kDa TAT-β-gal fusion protein to rapidly transduce into cells in vitro and become refolded into the enzymatically active form.

As a first analysis for in vivo transduction of TAT-β-gal protein, TAT-β-gal and control β-gal proteins were labeled with FITC and injected it I.P. into mice. In agreement with the TAT-FITC peptide results above, flow cytometry analysis of blood cells isolated 30 and 60 min post-I.P. injection demonstrated the presence of TAT-β-gal-FITC protein in all blood cells (Fig. 20D). Analysis of splenic cells 120 min post-I.P. injection also revealed the transduction of TAT-β-gal FITC protein into all cells present in the spleen (Fig. 20E). I.P. injection of control β-gal-FITC

labeled fusion protein (minus the 11 amino acid transduction domain) did not transduce into either blood or splenic cells (Fig. 20D-E). In addition, the reduced fluorescent signal from TAT-β-gal-FITC protein treated mice compared to TAT-FITC peptide treated mice was likely due to both decreased concentrations and labeling efficiencies of the former. These observations demonstrate the transduction of 120 kDa TAT-β-gal protein into all cells present in the blood and spleen in vivo.

Figures 20A-E are explained in more detail as follows. (20A) Structure of TAT-beta-gal and control beta-gal fusion proteins. (20B) Immunoblot analysis and enzymatic activity (20C) of cells treated with TAT-beta-gal (open squares) or B-gal (filled circles) proteins. (20D) Flow cytometry analysis of whole blood cells (30 min.) and splenocytes (20E, 120 min.) following I.P. injection into mice with TAT-beta-gal-FITC or control B-gal-FITC proteins.

Mice injected with TAT-β-gal protein for enzymatic activity in various tissue sections were analyzed next. Tissue samples from liver, kidney, heart muscle, lung and spleen were isolated at 4 and 8 hr post-I.P. injection, sectioned (10 and 50 μm) and assayed for β-gal activity by X-gal staining (Fig. 21A-21B). Both liver and kidney showed significant and universal β-gal enzymatic activity across the tissue sections at 4 and 8 hr post-I.P. injection. Heart muscle also showed strong β-gal activity throughout the muscle fibers. Sections from control β-gal injected mice showed only minor, sporadic staining that likely resulted from lymphatic uptake of control β-gal protein from the peritoneum. In addition, the control kidney staining presumably reflects the clearance of control β-gal protein from the blood stream. We also detected strong β-gal activity in lung sections (Fig. 21B). Unexpectedly, we noted differential regions of β-gal enzymatic activity within the spleen (Fig. 21B). Strong activity was detected in red pulp regions and significantly weaker β-gal enzymatic activity associated with white pulp areas, which are principally comprised of B and T cells. This observation

was at odds with the FACS results showing transduction of TAT-β-gal-FITC protein into all cells present in the spleen (Fig. 21C). However, previous reports of others have documented an uncharacterized β-gal inhibitory activity present in T and B cells (Krall and Braun *New Biol* (1992) 4:581; Elefanty et al., *Proc. Natl. Acad. Sci USA* (1998) 95:11897). Thus, the splenic results effectively act as an internal tissue control for β-gal activity.

Figures 21A-B are more specifically explained as follows. Analysis of b-gal enzymatic activity (X-gal staining) in liver (4hr), kidney (8 hr), heart muscle (4 hr) and spleen (4 hr) tissue sections from mice I.P. injected with TAT-beta-gal or control p-gal proteins, as indicated. Note weak beta-gal activity in white pulp region of spleen.

Next, brain sections from mice at various times post-I.P. injection of TAT-β-gal and control β-gal proteins were analyzed for enzymatic activity (Figs. 22A-C). Consistent with the rate of uptake from the peritoneum, blood flow and concentration-dependent nature of protein transduction, 2 hr brain sections showed low penetration of TAT-β-gal protein into brain tissue that was localized around blood vessels with minimal activity present in the surrounding parenchyma (Fig. 22A). However, by 4 hr post-I.P. injection, all regions of the brain had reached near equilibrium for β-gal activity. Mice injected with control β-gal protein showed no β-gal staining in the brain at 4 hr (Fig. 22A) or 2 and 8 hr. Strikingly, by 8 hr post-I.P. injection of TAT-β-gal protein, the β-gal activity resided primarily in the nuclei of cell bodies throughout all regions of the brain and to a lesser extent in the surrounding white matter (Fig. 22A). Though not observed in the liver, kidney, heart muscle, lung and splenic sections, perhaps due to higher local concentrations, these observations are consistent with the presence of a nuclear localization signal embedded in the 11 amino acid TAT protein transduction domain (RKKRR). Furthermore, the blood:brain barrier remained intact in TAT-β-gal treated mice as measured by the absence of

extravasated co-injected Evan's blue-albumin complexes in brain sections (Fig. 22B). In addition, a low magnification coronal brain section revealed β-gal activity in cell bodies throughout the brain of TAT-β-gal treated mice 8 hours post-injection (Fig. 22C).

Figures 22A-C are more particularly explained as follows. Transduction of TAT-beta-gal protein across the blood:brain barrier. beta-gal enzymatic activity (X-gal staining) in brain sections from mice I.P. injected with TAT-beta-gal or control beta-gal proteins at indicated times. Note localization of TAT-beta-gal to cell bodies-nuclei throughout the brain section at 8 hr post-I.P. injection.

It is believed that the present observations represent the first demonstration of introducing a large, biologically active protein across the blood:brain barrier and into all other tissues assayed in a mammal. It is believed that the observations can be employed with other animals including other rodents, domesticated animals, primates and especially human subjects.

Such ability to systemically deliver biologically active and therapeutic proteins efficiently and effectively to all tissues, including the brain, will undoubtedly impact treatment of a wide variety of diseases. Furthermore, in cases such as cancer where only specific cell populations are affected, PTD domains can be used to link toxic proteins such as p53 and p19ARF and administered systemically, inducing apoptosis only in cancerous cells while leaving normal cells intact. Thus, in this way, PTD fused peptides may be utilized to effectively control diseases which to this day, have been difficult to treat, such as metastatic cancer.

More generally, the transduction of peptides and proteins into mice reported in the present example provide a solid framework for epigenetic complementation experiments in model organisms and eventual transduction of therapeutically relevant proteins into patients in

the form of protein therapy. In addition, protein transduction allows access to the built-in evolutionary specificity of biologically active full length proteins that may avoid non-specific interactions associated with small molecule therapies while dramatically reducing the effective dose. Other important questions that arise are the potential immunogenicity resulting from multiple administrations of transducing proteins and any toxicity associated with long-term transduction of proteins into tissues, especially the brain. Clearly, these parameters, including the half-life and effective doses, will be dependent, e.g., on the nature of the protein being transduced, though most may well be comprised of >99% human or same species origin (less the PTD sequence) and therefore, likely not presented on the MHC. In addition, we have injected a mouse with 1 mg/kg of body weight of a TAT PTD fusion protein each day for 14 consecutive days and observed no obvious signs of gross CNS/PNS problems or systemic distresses. Indeed, the mouse even gained weight on schedule with his siblings.

As discussed in the section above entitled "Summary of the Invention" the "Blue" mouse has a number of important uses and advantages including use in the detection and characterization of pharmacologically useful molecules including vaccines, drugs, and other medicinals. That is, the "Blue" mouse can be used as a convenient animal model to test a desired fused molecule in a whole animal setting. More particular reference to a "Blue" mouse means that the mouse exhibits detectable expression (protein fusion) or presence (non-protein fusion, e.g., a drug or small molecule) in at least liver, kidney, lung, heart muscle, and preferably spleen tissue. Methods for detecting that expression or presence are known in the field and include those specific methods discussed herein.

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9. Human Jurkat T cells and HepG2 cells were maintained as described (4,6). Flow Cytometry (FACS; Becton Dickinson) was performed as described (4). FACS kinetic analysis was performed by injection of 20,ul of PTD-FITC-labeled peptides directly into a sample undergoing FACS analysis. Confocal microscopy was performed on peptide treated Jurkat cells fixed in 4% paraformaldehyde.
10. Over forty 4-8 week old C57BL/6 and 129 mice were injected intraperitoneally (I.P.) with 1.7 nmol of TAT-t'l'l'C peptide in 500 p1 of PBS or 100-500 microgram of TAT-beta-gal and beta-gal control protein present in 0.5-2.0 ml PBS/10% glycerol. Blood was isolated from the orbital artery and splenocytes were isolated by the frosted slide method at indicated time points. Animals were sacrificed tissues harvested and frozen in O.C.T. media. 10-50 micron sections were cut on a cryostat, fixed in 0.25% gluteraldehyde for I 5 min and developed as required in 0.2% X-gal solution (11) or analyzed by fluorescent confocal microscopy. All animal procedures were performed in accordance with institutional guidelines.
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Example 18- Enhanced Transduction Using Synthetic Transduction Domains.

As discussed above, it has been found that significant improvements in the transduction efficiency can be achieved by generation of recombinant in-frame fusion proteins expressed in bacteria containing the minimal 11 amino acid transduction domain from HIV TAT (aa 47-57) combined with a denaturation protein purification protocols. Indeed, the inventors have made and transduced over 50 proteins from 15 to 120 kDa, including: transcription factors, transcriptional regulators, viral and cellular sequestering proteins, enzymes, caspases, proteases and GFP (green fluorescent protein). In addition, fusion proteins and peptides containing either an N' or C' terminal TAT domain transduce into all cell types assayed in vitro thus far, including: peripheral blood lymphocytes, diploid fibroblasts, fibrosarcoma, osteoblasts, osteoclasts, bone marrow stem cells, macrophage, NIH 3T3, HeLa, Jurkat T cells, HepG2, gliomas and all cells present in whole blooded. These results suggest that nearly any fusion protein can be efficiently transduced into a wide spectrum of many organ, tissue and cell types.

There has been little recognition about the structural requirements for protein transduction. In the present example, we have specifically discovered that the modeled structure of the TAT transduction domain has a strong alpha helical character with a face of arginine residues. Accordingly, a series of non-naturally occurring protein transduction domain (PTD) peptides that show significant enhancement of protein transduction potential into cells both in vitro and in vivo were made.

The following work extends previous results and discussion including those of Example 10 above.

1. Modeled structure of TAT transduction domain

There is understanding that the region of HIV TAT encompassing amino acids 47-57 (Fig. 23A) can transduce across cellular lipid bilayer membranes in a receptor-independent fashions. However, the sequence requirements for transduction mediated by TAT remain unknown and the crystal structure has not been solved. The molecular structure of the TAT domain was modeled using the LINUS protein structure and GRASP molecular surface predictive programs. We found that this region of TAT has strong alpha helical characteristics (light blue ribbon backbone) (Fig. 23B). Rotation of the helix by two 90° right-handed rotations shows that the TAT domain appears to be comprised of an amphipathic helix, with the Arg basic charges at the left and the hydrophobic surfaces at the right of the helical axis. Of striking note, is the alignment of Arg residues (dark blue) present on the entire length of one face of the predicted alpha helix (Fig. 23C). These models suggest that the TAT transduction domain forms a strong amphipathic alpha helix with a face of basic Arg residues.

Figures 23A-C are more particularly explained as follows. Fig. 23A: Primary sequence of minimal HIV TAT protein transduction domain (aa 47-57; single amino acid code). Fig. 23B: Modeled molecular surface and peptidyl bond of the 11 amino acid HIV TAT protein transduction domain using the LINUS and GRASP programs (see methods section below). Orientation is N terminus bottom, C terminus top, and rotations are 90° and l 80° along vertical axis of helix. Basic surface regions of Arg residues in dark blue, helical peptidyl backbone shown in light blue ribbon. Fig. 23C Alpha helical wheel of TAT transduction domain sequence.

2. Synthetic protein transduction domains (PTDs)

A series of synthetic, non-naturally occurring PTD peptides (Fig. 24) were made. In general, the PTD peptides fall into two groups: 1.)

peptides with optimized Arg alignment on one face of the helix while strengthening the alpha helical value by substitution with Ala residues (PTDs 3-6) and 2.) peptides consisting of an Arg cylinder (PTDs 7 & 8). As discussed, the terms "SFD" and "PTD" are often used interchangeably herein. Compare Figs. 7 and 24. Group I PTD peptides are further delineated by both optimization of the Arg face and reduction of the extent of Arg around the helical cylinder (Fig. 24).

Figure 24 is explained in more detail as follows. Predicted alpha helical wheels of TAT and non-naturally occurring Protein Transduction Domains (PTD) (single letter amino acid code). Numbering indicates sequential amino acid position; values in parentheses represent fold change of FITC emission normalized to TAT peptide (= lx)(see Fig. 3a)

Ala residues were chosen to substitute for naturally occurring non-Arg TAT residues based on the highest alpha helical stabilizing value (-0.77 kcal/mol) while Arg residues are the second most stabilizing (-0.68 kcal/mol). Each PTD peptide was synthesized with an N' terminal FITC-Gly residue followed by a Gly-Gly-Gly motif. Due to a >99% coupling efficiency during peptide synthesis, this allowed for direct quantitative comparisons between each PTD peptide based on fluorescent values. Prior to use on cells, all peptide concentrations were normalized by fluorometeric analysis and diluted accordingly.

To analyze the transduction potential of each peptide, Jurkat T cells were treated with normalized TAT and PTD peptides for 30 min at 37°C. Peptide treated cells were then analyzed by flow cytometry (FACS) analysis and compared to the auto-fluorescence of untreated cells and control cells treated with an equal molar amount of free FITC (Fig. 25A). Consistent with previous work, about 100% of cells in the population were transduced by TAT peptide. In addition, the similar FACS peak widths before (auto-fluorescence) and after TAT peptide treatment suggested that all cells in the treated population have a near identical intracellular concentration of TAT peptide (Fig. 25A).

Comparison of the transduction potential of PTD peptides to TAT peptide by FACS analysis identified multiple levels of increased transduction potential. Substitution of alpha helical promoting Ala residues at three positions opposite the Arg face of PTD-3 peptide, while maintaining five Arg residues (TAT has six), resulted in a significant increase (5x) in transduction potential compared to TAT peptide (Fig. 25A). Additional substitution with Ala residues, while maintaining the Arg content and distribution of PTD-3, yielded PTD-5 peptide that resulted in a further enhancement of the transduction potential (8x) (Fig. 25A). Strikingly, further strengthening the putative alpha helix with Ala residues while limiting the Arg content to three closely aligned residues down the face of the helix dramatically improved the transduction potential of PTD-4 peptide compared to the original TAT sequence (33x) (Fig. 25A). In contrast, PTD peptides containing entire cylinders of nine or seven Arg residues, PTD-7 and PTD-8, resulted in near identical transduction potentials compared to TAT peptide (Fig. 24).

Fluorescent confocal microscopy of treated cells confirmed the rank order of transduction potentials and most importantly, demonstrated the presence of PTD peptides and TAT peptide within the cytoplasm and nucleus of cells, whereas control FITC was merely bound to the cellular membrane (Fig. 25B). Thus, the observed dramatic enhancements in transduction potential of PTD peptides compared to TAT peptide by both closely aligning the Arg residues and further strengthening the putative alpha helix with Ala residues substitutions is consistent with the modeled alpha helical structure.

The kinetics of protein transduction into cells was also examined. Previous work had shown that cells treated with transducing peptides and proteins achieved maximum intracellular concentration in less than 10 min. Therefore, a kinetic (real time) FACS analysis of cells treated with either PTD-4 peptide or TAT peptide was done (Fig. 25C). The baseline auto-fluorescence of Jurkat T cells (1 x 10^6 cells/0.7 ml) was

pre-determined followed by injection of 10 pi of PTD-4 peptide or TAT peptide into the tube during continuous FACS analysis. Notably, both PTD-4 and TAT peptide treated cells reached maximum intracellular concentration in less than 30 sec in an apparent first order rate constant for transduction. However, consistent with the above histogram analysis (Fig. 25A) and confocal microscopy, PTD-4 peptide achieved a significant increase in intracellular concentration compared to TAT peptide (Fig. 25C). Thus, PTD peptides transduced into ~100% cells in an extremely rapid fashion with significant enhancement of transduction potential compared to TAT peptide.

Figures 25A-C are explained in more detail as follows. Characterization of PTD-FITC peptides in vitro. Figure 25A: Flow cytometry comparison of untreated and treated Jurkat T cells with equal molar amounts of control FITC and TAT, PTD-3, PTD-4 and PTD-5 peptides. Normalized fluorescent values are to TAT peptide (= 1 x) (right panel). Figure 25B: Fluorescent confocal microscopy of cells from (Fig. 25A) demonstrated transduction of TAT, PTD-4 and PTD-5 peptides into cells, whereas control free FITC was merely attached to the cellular membrane. Note, fluorescent intensity values adjusted to view each treated cell population. Fig. 25C, Kinetic (real time) flow cytometry analysis of Jurkat T cells after injection (arrow) of 10 microliters of a 100 nM PTD-4 peptide (red) or TAT peptide (blue).

4. Transduction of PTD-4 peptide and PTD-4-GFP protein into the Mouse

The ability to transduce TAT peptides and fusion proteins into mice was also examined. These results confirm and extend those discussed above including those provided by Example 17 above

As discussed, extremely efficient transduction of PTD-4 peptide into cells in vitro was found. The in vivo ability of PTD-4 peptide to transduce into cells in a mouse model was also analyzed. Administration via several routes was determined to be appropriate with delivery via an

intraperitoneal injection (I.P.) being particularly preferred. Without wishing to be bound by theory, the fusion protein may be taken-up by several mechanisms, including: the lymphatic system which drains the peritoneal cavity, direct transduction across the peritoneum into the blood stream and direct transduction into to organs present in the peritoneal cavity.

C57BL/6 mice were I.P. injected with 0.6 nmol of PTD-4 peptide, TAT peptide or control free FITC in 500 HI PBS. Whole blood was isolated from the orbital artery 30 min post-I.P. injection and analyzed by FACS (Fig. 26A). Surprisingly, both PTD-4 peptide and TAT peptide treated mice demonstrated transduction into ~100% of cells present in whole blood compared to untreated control mice. No further increases were observed with either PTD-4 or TAT peptides at 60 min post-I.P. injection. Consistent with the enhanced in vitro transduction potential shown above in previous examples, PTD-4 peptide showed a significant increase (5x) in intracellular concentration of whole blood cells compared to TAT peptide (Fig. 26A). Control FITC injected mice showed no increase in background fluorescence of blood cells. Thus, PTD-4 peptide has an increased transduction potential both in vitro and in vivo.

The following discussion confirms and extends results of Example 14 above and Figures 17A-D. Here, the ability of PTD-4 peptide to transduce into various tissues of mice was observed. Skeletal muscle and brain tissues were dissected, frozen and sectioned from C57BL/6 mice 30 min post-I.P. injection with PTD-4 peptide or control free FITC (Fig. 26B). Fluorescent confocal microscopy of skeletal muscle (quadriceps) showed significant fluorescent signal compared to controls throughout the sections (Fig. 26B). Delivery of molecules across the blood:brain barrier and into the brain parenchyma remains a significant boundary to compounds, peptides and proteins. Remarkably, fluorescent confocal microscopy of 10 μm brain sections from PTD-4 peptide treated mice showed a strong fluorescent signal throughout the entire brain compared to control sections. Thus, the non-naturally occurring PTD-4 peptide has

a dramatically enhanced potential to transduce into cells both in vitro and in vivo, including across the blood:brain barrier.

Figures 26A-B are explained more specifically as follows. Characterization of PTD-4-FITC peptide in vivo. Fig. 26A: Flow cytometry analysis of whole blood cells from mice 30 min post-I.P. injection with PTD peptide, TAT peptide, control free FITC or untreated control, as indicated. Fig. 26B: Fluorescent confocal microscopy of skeletal muscle (top panels) and brain (bottom panels) tissue sections from mice 30 min post-I.P. injection with PTD-4 peptide or control free FITC. Note, depleted areas of fluorescent intensity due to "freezer burn" of section and thus, absence of tissue in those regions of the section.

The ability of PTD-4 vs. TAT to deliver the Green Fluorescent Protein (GFP, 27 kDa) into blood cells of mice was also compared. PTD-4-GFP and TAT-GFP fusions were generated, expressed in bacteria and purified. See reference 6 below. Both PTD-4-GFP and TAT-GFP proteins were found to transduce into cells in vitro by fluorescent confocal microscopy (Fig. 27A) and FACS. C57BL/6 mice were I.P. injected with 500 microliters of each protein in 1ml PBS. Whole blood was isolated from the orbital artery at 60 min post-I.P. injection and analyzed by FACS (Fig. 27B). Mice injected with TAT-GFP protein showed only a minor increase above the background auto-fluorescence of whole blood cells. However, mice injected with PTD4-GFP protein showed a significant transduction into ~ 100% of whole blood cells compared to untreated control mice (Fig. 27B). In addition, injection of control GFP protein (minus the 11 aa transduction domain) showed no increase in fluorescence of whole blood cells (Fig. 27B). Thus, the PTD-4 transduction domain has an increased potential to deliver both peptides and proteins in vitro, including across the blood:brain barrier.

Figure 27A-B are explained in more detail as follows. Characterization of PTD-4-GFP protein in vitro and in vivo. Fig. 27A Fluorescent and phase confocal microscopy of Jurkat T cells transduced

with TAT-GFP or PTD-4-GFP proteins. Fig. 27B: Flow cytometry analysis of whole blood cells from mice 60 min post-I.P. injection with TAT-GFP protein, PTD-4-GFP protein, control GFP protein or untreated control, as indicated.

The foregoing results demonstrate, both in vitro and in vivo, that optimization of Arg residues plus substitution with alpha helical promoting residues (Ala) resulted in dramatic enhancements of protein transduction potential. This result presents several important advantages including providing for significant dose reductions. In addition, these observations are entirely consistent with the modeled alpha helical structure of the TAT domain..

The following methods were used as needed in this example.

a) Cell culture and flow cytometry analysis.

Human Jurkat T cells were maintained in RPMI plus 5% fetal bovine serum (FBS), penicillin and streptomycin in 5% CO2 at 37°C as described below. 1 x 10^6/ml Jurkat cells were treated with FITC-labeled peptides at 37°C and 10,000 cells assayed by Flow Cytometry (FACS; Becton Dickinson) at indicated times. For kinetic FACS analysis, Jurkat T cells (1x10^6/ 0.7 ml) were placed in polypropylene FACS tube in an ice bath and the cellular auto-fluorescence normalized. 10 ul of FITC-labeled peptides or control FITC were then injected directly into the tube during continuous FACS analysis. Fluorescent confocal microscopy was performed on treated Jurkat T cells fixed in 4% paraformaldehyde.

b) PTD peptides and GFP fusions.

Peptides were synthesized containing an N' terminal synthetic FITC-Gly residue that resulted in a near 100% coupling of FITC to the synthesized peptide. The FITC-Gly N' terminal residue was followed by 3x Gly residues and then the 11 aa TAT (residues 47-57) or 11 aa synthetic protein transduction domains (PTD) listed in Fig. 24. After synthesis and HPLC purification, all peptides were resuspended in water. Due to the N' terminal FITC-Gly coupling efficiency (>99%) to all peptides during

synthesis, peptide concentrations were normalized by fluorescence values from a Fluorometer. PTD-4-GFP and TAT-GFP were generated by placing the GFP open reading frame into pPTD-4, a derivative of pTAT6 that substitutes the PTD-4 sequence for TAT, or pTAT. Fusion proteins were expressed in BL21 (DE3)LysS cells (Novagen) and purified in 8 M urea, followed by desalting into PBS as described. Control GFP protein contained the same N' terminal leader minus the 11 amino acid protein transduction domain.

c) Modeled TAT 47-57 structure.

Structural predictions of the TAT (47-57) protein transduction domain were made using the LINUS programs. The molecular surface of the predicted structure was then calculated and displayed using the GRASP programs.

d) Mice

4-8 week old C57BL/6 mice (~20 g) were injected intraperitoneally (I.P.) with 0.6 nmol of TAT-FITC peptide, PTD-FITC peptides or control free FITC in 300-500 Al of PBS. Blood was isolated from the orbital artery at indicated time points. 2 microliters of whole blood was diluted with 3 microliters of PBS and the entire 5 microliter sample was analyzed by FACS. Animals were sacrificed, tissues harvested and frozen in O.C.T. media. 10 micron sections were cut on a cryostat, fixed in 4% buffered formalin, mounted with anti-fade (Mol. Probes, CA) and analyzed by fluorescent confocal microscopy. All animal procedures were performed in accordance with institutional guidelines.

The following references and discussion are incorporated herein by reference.

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Example 19-Anti-Microbial Protein Transduction Strategy

There has been recognition that worldwide resistance to current antibiotics treatments is growing and represents a difficult problem to overcome. The number and types of new antibiotics is decreasing

dramatically, while resistance to currently still effective antibiotics is more and more prevalent. It is clear to most in the biomedical field that in the absence of a new strategy, current antibiotics will be useless sometime in the next century. There is a need in the field to find compositions and methods that can return current antibiotics and other therapeutic molecules to the caregiver's armamentarium of medicinal agents.

To address this need, a anti-microbial strategy is proposed in accordance with this invention. In particular, the strategy is based on protein transduction that specifically kills bacteria while leaving human/mammalian cells unharmed. The strategy incorporates several properties of protein transduction to obtain this specificity.

To illustrate this aspect of the invention, a model system was constructed to make a transducible bacterial β-galactosidase fusion protein containing an N-terminal Protein Transduction Domain (PTD-β-gal) (Figure 28A). PTD-β-gal protein transduces into human cells in vitro very rapidly, as do all PTD fusion proteins we have generated (Figure 28B). However, the refolding rate, as measured by the ability of PTD-β-gal to convert the X-gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) from a clear, soluble compound into a blue, insoluble one, lagged significantly behind the transduction rate (Figure 28B). Indeed, PTD-β-gal had reached maximum intracellular concentration in less than 15 minutes, while β-gal activity was still increasing at 2 hours post-addition of PTD-β-gal to the cells. A negative control of β-gal minus the PTD showed no ability to transduce into cells or to cleave X-gal in vivo. Thus, the ability to refold a bacterial protein, not a protein produced in bacterial, but a protein encoded by a bacterial gene, was very inefficiently refolded by the human HSP90 chaperones that refold misfolded proteins post-translationally.

Second, denaturation of PTD fusion proteins, such as high concentrations of urea (8M urea), result in an increased transduction potential (see Nagahara et al. *Nature Medicine* 4:1449-1452 (1998)). However, that is not to say that correctly folded or soluble mammalian and viral PTD fusion proteins produced in bacteria are unable to transduce, they just appear to have a reduced capacity to elicit a biological response. Thus, correctly or near correctly folded, soluble in-frame PTD fusion proteins are capable of transducing into cells.

Denaturation of many different mammalian or viral PTD fusion proteins results in transduction and refolding inside mammalian cells, likely by HSP90 chaperones has been shown. See Ezhevsky et al. 1997; Lissy et al. 1998; Nagahara et al. 1998; Vocero-Akbani et al. 1999 (supra). The ability of a bacterial PTD fusion protein (i.e. encoded from a bacterial gene, not a mammalian one) to transduce and be refolded in human cells after being denatured by 4 or 8M urea was compared. Due to the ability to convert X-gal into a blue insoluble form, PTD-β-gal was selected as the model fusion protein.

PTD-β-gal was purified from BL21 (DE3) LysS-expressing bacteria by sonication as previously described (Nagahara et al. 1998), with the exception that bacterial pellet was split into equal parts and sonicated in PBS, 4M urea or 8M urea. The different sonicates were purified over a Ni-NTA column, dialyzed against PBS and concentrations normalized. Each PTD-4-β-gal prep was added to human HepG2 hepatocellular carcinoma cells and assayed for: 1) intracellular concentration of PTD-β-gal by anti-β-gal immunoblot; and 2) assayed for β-gal enzymatic activity by washing the cells in PBS, fixing in 2% gluteraldehyde for 15 minutes, and then incubation at 37°C with a PBS/10%DMF solution containing 0.2% X-gal substrate. Cells treated with PTD-β-gal from the PBS (Figure 28B) and 4M urea sonicated preps retained the ability to transduce and to be refolded. However, even though all other mammalian and viral proteins can be sonicated in 8M urea and refolded after transduction into cells to become active, sonication of PTD-β-gal protein in 8M urea completely

inactivated the enzymatic activity of this protein, even though it was still able to rapidly transduce into the cells (Figure 28B).

These observations demonstrate that proteins encoded by mammalian and/or viral genes that are denatured, such as 8M urea treatment, are capable of being recognized and efficiently refolded by human chaperones, such as HSP90. However, proteins encoded from bacterial genes are NOT recognized by human/mammalian chaperones as substrates for refolding. Without wishing to be bound to theory, one basis for discrepancy lies in the observation that the short amino acid refolding sequence "flags" that mammalian HSPs recognize in human proteins are not present in bacterial proteins produced from bacterial genes. However, bacterial HSPs obviously do recognize the amino acid refolding sequence "flags" present from bacterially-encoded gene products.

This observation allows for the generation of anti-microbial/bacterial transducing proteins that bind, target and inactivate various proteins involved in the bacterial DNA replication, protein translation, membrane integrity, etc. by using denatured transducible bacterial proteins produced from bacterial gene sequences. Thus, denatured PTD-bacterial fusion proteins will be refolded ONLY in prokaryotic cells and thus effect a toxic killing response, whereas the human cells are unable to refold the denatured bacterial protein and will remain unharmed.

For example, the invention can be used to transduce a wide spectrum of anti-microbial agents into pathogenic or potenially pathogenic microbes. More specific examples include vaccines, bacteriocidal/bacteriostatic proteins, antibiotics such as penicillin, sulfa drugs, ect. See Gilman, et al. *supra* and The Merck Manual 16th Ed. *General Medicine: Infectious Agents* (1992) (Merck Research Laboratories) (Rahway, N.J) for other examples of pharmacological agents that have particularly succumbed to emergence of resistant microbial strains.

Significantly, this invention provides for renewed use of these previously beneficial molecules.

In summary, the observation above allows for the further discrimination or increased specificity of targeting prokaryotic cells for death from human/mammalian cells in the whole organism.

Example 20- New Molecule Discovery Strategies

The foregoing discussion and examples show that it is possible to transduce a wide spectrum of molecules into desired cells, tissue and organs. Also demonstrated is good transduction of essentially an entire mammal. In particular, it has been shown that practice of this invention can substantially improve bioavailability and provide a highly preferred means of introducing therapeutic molecules into those cells, tissues, organs up to essentially the entire mammal.

More specifically, the invention provides means of enhancing the bioavailablity of very large molecules such as proteins as well as smaller molecules, e.g., those having a molecular weight of a few hundred daltons. Such molecules often possess one or more characteristics that prevent efficient bioavailability. For example, the large size of many proteins can impede bioavailablity. Many small molecules have significant hydrophobicity, hydrophilicity, solubility or related impediments that can decrease of even block good uptake.

Related to these problems is the recognition by the inventor that many molecules with substantial therapeutic value are being lost. More specifically, it is believed that more than half of the molecules analyzed by conventional drug screening methods are discarded due to unfortunate constraints imposed by the screen such as size, hydrophobicity, hydrophilicity, charge, solubility, and the like. The present invention solves this problem by providing new molecule discovery strategies that use compositions and methods of this invention to screen these molecules.

As an illustration of this aspect of the invention, the forgoing discussion and examples have already shown that the bioavailability of several molecules was improved. For example, bioavailablity of FITC was significantly improved when coupled to TAT PTD or to any one of the modified PTDs disclosed herein. For More particularly, when FITC was coupled to TAT PTD, PTD-4, and PTD-5 and transduced into Jurkat T cells, fluorescent confocal microscopy of treated cells demonstrated the presence of PTD peptides and TAT peptide within the cytoplasm and nucleus of cells, whereas control FITC was merely bound to the cellular membrane. See Example 18 above and Figure 25B. Thus, by coupling FITC, which has no bioavailability alone, to TAT PTD or to any one of the modified PTDs, FITC was very efficiently transduced into cells.

See Example 18 and Figure 26A above in which the PTD-4 engineered peptide enhanced bioavailability of FITC when compared to the TAT peptide.

See also Figures 19A-D and the corresponding example.

1. Molecule Discovery *in vitro*

As discussed, the present invention can be used to transduce a wide spectrum of large and small molecules into cultured cells. Thus it is an object of this invention to provide a new molecule discovery strategy that can be used to effectively screen molecules that heretofore have been discarded do to unacceptable hydrophobicity, hydrophilicity, charge, or size. The strategy can be used to augment or replace prior drug screening protocols as required.

A wide variety of eukaryotic cells are suitable for use in the molecule discovery strategy. Preferred are animal cells that are routinely used in conventional drug screening methods. Illustrative cells include HeLa, Cos, CV-1 or other suitable cells recognized as being useful in this field. Also contemplated is use of primary cells including tissue samples

and organs obtained from a primate and particularly a human subject. However, for many applications of the in vitro molecule discovery strategy, well-known immortalized animal cells will often be preferred.

Optimal practice of the method involves making a desired transduction construct that includes at least one molecule to be screened, contacting suitable animal cells with the construct under conditions sufficient for transduction, and transducing the construct into the cultured animal cells. Specific screening strategies will be informed by several recognized parameters including the molecule screened, the cultured cells used, and the pharmacological activity of interest. For example, transduced cells can be tested for appearance or loss of a particular cell phenotype including selection with cytotoxic drugs. Also contemplated are screens involving immunological and/or molecular techniques e.g., antibody screening including ELISA and Western blots, biopanning, radiolabeling techniques, detection of RNA including Northern blots and detection of DNA including PCR and DNA hybridization techniques.

2. Molecule Discovery *in vivo*

In many instances, the aforementioned in vitro screens will be used to select candidate molecules for further in vivo testing. In particular, molecules giving good activity in any of the in vitro screens including the molecule discovery strategy can be additionally tested in the present in vivo screening strategy.

In one embodiment, a molecule exhibiting good activity in the in vitro molecule discovery strategy can be transduced into essentially an entire subject animal, e.g., a mouse, by as described previously. Here, the molecule is additionally tested at the organismal level which testing can determine a variety of important parameters such as tolerance, stability and/or clearance and degradation. This feature of the invention guards against selection of molecules which may have undesirable side effects or which may be converted into harmful by-products.

A more specific example of the present in vivo molecule discovery strategy follows.

There is recognition that cyclosporins A, B, C, D and G are nonpolar cyclic oligopeptides with good immunosuppressant activity but with minimal solubility in water and other pharmaceutically acceptable vehicles. See, e.g., *The Merck Index*, 11ed (1989) Merck & Co. (Rahway, NJ) pp. 2761-2762. In accord with this invention, each cyclosporin can be covalently attached to any one of the transduction proteins disclosed herein including those preferred PTD constructs providing good bioavailability of FITC, ect. Choice of a specific covalent attachment and the transducing protein will be guided by several parameters including intended use.

In a more particular embodiment, cyclosporin A can be covalently attached to PTD-4 to provide for good transduction of the molecule into cells in vitro. Alternatively, the cyclosporin A can be spaced from the PTD-4 by any of the linker sequences discussed herein including those having proteolytic sites. In this embodiment, the cyclosporin A is released following transduction into cells that have (or can be induced to have) the requisite protease. In another embodiment, the cyclosporin A can be covalently linked to the PTD-4 through a disulfide bond linker that includes at least one disulfide (-S-S-) bond. In this example of the invention, transduced cyclosporin A is desirably released inside the reducing environment typical of most cells.

More specifically, cyclosporin A can be covalently attached to the PTD-4 protein (or other suitable transducing protein) through about one disulfide linkage. Attachment to the cyclosporin A molecule is preferably made through an amino acid or other suitable linker sequence which does significantly reduce immunosuppressant activity of the molecule. In this illustration of the invention, a preferred molecule is covalently linked in sequence: cyclosporin A \ linker \ -S-S- \ PTD-4. The linker

can be any acceptable length as described herein including about 5 to about 10 amino acids. Attachment to the PTD-4 molecule can be at the N or C terminus as needed.

A amount of the construct sufficient for transduction (see above discussion and Examples) is administered to suitable animal cells in vitro. Preferred are murine cells which are immunologically incompatible with a recognized mouse model having a robust immune system. In this example of the invention, transduction of the cyclosporin A into the murine cells can be monitored by one or a combination of different strategies including radiolabeling the cyclosporin A prior to or concurrent with attachment to the PTD-4. Murine cells effectively transduced by the fusion construct will release cyclosporin A inside the cells by cleavage of the disulfide bond.

Bioactive cyclosporin A inside the murine cells can be monitored by employing conventional graft rejection technology. In this example, murine cells suitably transduced with the cyclosporin A are grafted onto a mouse host which is known to be immunologically incompatible with the murine cells, ie., they will be rejected. A significant reduction or elimination of those murine cells that have the free cyclosporin A can be monitored by one or combination of techniques known in the graft rejection field including immunological techniques.

As will be appreciated, this embodiment of the invention is particularly useful for developing and screening for cyclosporins with improved activity.

All references disclosed herein are incorporated by reference in their entirety.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make

modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. A fusion molecule comprising at least one protein transduction domain (PTD) and at least one linked molecule, wherein the linked molecule is suspected of having or has recognized capacity to treat or prevent a medical or veterinary condition in a subject mammal.

2. The fusion molecule of claim 1, wherein the subject mammal is a human patient and the linked molecule is a vaccine, anti-infective drug, cardiovascular drug, anti-tumor drug, analgesic, anti-inflammatory, diagnostic marker or a drug for treatment or prevention of a nervous system disorder.

3. The fusion molecule of claim 2, wherein the nervous system disorder is a disorder of the central nervous system (CNS) or the peripheral nervous system (PNS).

4. The fusion molecule of claim 3, wherein the CNS disorder is one of Alzheimer's disease, Parkinson's disease, Huntington's disease, pre-senile dementia, epilepsy and especially seizures, compulsive behavior, meningitis including virally and bacterially induced meningitis, encephalitis, ischemia, scrapie or related degenerative disorders of the brain, dyslexia, age-related memory loss, or Lou Gehring's disease.

5. The fusion molecule of claim 2, wherein the vaccine has or is suspected of having capacity to treat or prevent a bacterial or viral infection.

6. The fusion molecule of claim 5, wherein the vaccine includes at least one protein from a human DNA or RNA virus.

7. The fusion molecule of claim 6, wherein protein is a coat protein and the human DNA virus is a papillomavirus, hepatitis virus, cytomeglovirus or herpes virus.

8. The fusion molecule of claim 6, wherein protein is a coat protein and the human RNA virus is HIV or AIDS-related virus.

9. The fusion molecule of claim 2, wherein the anti-infective agent has or is suspected of having capacity to treat or prevent infection by one or more pathogens.

10. The fusion molecule of claim 9, wherein the pathogens is at least one of a prion, bacterium, yeast, fungi, protozoa, helminth, nematode, or amoebae.

11. The fusion molecule of claims 9-10, wherein the anti-infective agent is an antibiotic.

12. The fusion molecule of claims 9-11, wherein the antibiotic is a sulfa drug, tetracycline, a quinolone, beta-lactam antibiotic including penicillin and cephalosporin, aminoglycoside, erthromycin; or a pharmaceutically acceptable derivative thereof.

13. The fusion molecule of claim 2, wherein the anti-tumor drug is an anti-metabolite, e.g, a pyrimidine analog, purine analog; vinca alkaloid, antibiotic, or a hormone or hormone antagonist, e.g, estrogen, androgen, ect.

14. The fusion molecule of claims 2-4, wherein the drug for treatment or prevention of the nervous system disorder is a recognized drug formulated for management of Alzheimer's disease, Huntington's disease or Parkinson's disease.

16. The fusion molecule of claims 1-15 further comprising covalently linked in sequence the protein transduction domain (PTD) fused to a peptide linker sequence which sequence is fused to the linked molecule.

17. The fusion molecule of claims 1-16 further comprising at least one disulfide linkage adapted to release the linked molecule after transduction of the fusion molecule.

18. The fusion molecule of claims 1-17, further comprising at least one protease cleavage site adapted to release the linked molecule after transduction of the fusion molecule.

19. The fusion molecule of claims 1-18, wherein the fusion molecule is formulated to complement a pre-existing genetic defect in the mammal.

20. The fusion molecule of claim 19, wherein the genetic defect is an unacceptable level of at least one cell component such as a protein, peptide, RNA or DNA.

21. The fusion molecule of any one of claims 1-20, wherein the linked molecule has or is suspected of having poor bioavailability relating to at least one of unacceptable: water solubility, lipid solubility, size, charge, biotransformation including plasma clearance.

22. The fusion molecule of any one of claims 1-21, wherein the fusion molecule has a molecular weight of at least about 500 to 600 Da.

23. The fusion molecule of any one of claims 1-22, wherein the fusion molecule is soluble in water or lipid.

24. The fusion molecule of claims 1-23, wherein the bioavailability of the linked molecule is known or suspected of reducing or preventing efficient entry of the fusion molecule into the central nervous system (CNS) of the mammal.

25. The fusion molecule of claims 1-24, wherein the linked molecule is experimental and has not been approved for clinical use.

26. The fusion molecule of claims 1-25, wherein the protein transduction domain comprises at least a peptide represented by the following formula: B_1 -X_1-X_2-X_3-B_2-X_4-X_5-B_3, wherein B_1, B_2, and B_3 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3 , X_4 and X_5 are each independently an alpha-helix enhancing amino acid the same or different.

27. The fusion molecule of claims 1-26, wherein the protein transduction domain comprises at least a peptide represented by the formula B_1 -X_1-X_2-X_3-B_2-X_4-X_5-B_3, wherein B_1, B_2, and B_3 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3 , X_4 and X_5 are each independently an alpha-helix enhancing amino acid the same or different.

28. The fusion molecule of claims 26 or 27, wherein at least one of the basic amino acids is arginine.

29. The fusion molecule of claims 26 or 27, wherein at least one of the alpha-helix enhancing amino acids is alanine.

30. The fusion molecule of claim 26 or 27, wherein at least one of the alpha-helix enhancing amino acids is alanine and the basic amino acids are arginine substantially aligned along at least one face of the peptide.

31. The fusion molecule of claims 1-30, wherein the protein transduction domain comprises at least a peptide represented by the following formula: B_1 -X_1-X_2-B_2 -B_3 -X_3-X_4-B_4; wherein B_1, B_2, B_3, and B_4 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3 , and X_4 are each independently an alpha-helix enhancing amino acid the same or different.

32. The fusion molecule of claims 1-31, wherein the protein transduction domain comprises the following formula: B_1 -X_1-X_2-B_2 -B_3 - X_3-X_4-B_4; wherein B_1, B_2, B_3, and B_4 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3, and X_4 are each independently an alpha-helix enhancing amino acid the same or different.

33. The fusion molecule of claim 31 or 32, wherein at least one of the basic amino acids is arginine.

34. The fusion molecule of claim 31 or 32, wherein at least one of the alpha-helix enhancing amino acids is alanine.

35. The fusion molecule of claim 31 or 32, wherein at least one of the alpha-helix enhancing amino acids is alanine and the basic amino acids are arginine substantially aligned along at least one face of the peptide.

36. The fusion molecule of claims 1-35, wherein the protein transduction domain comprises at least the following sequences: AGRKKRRQRRR (SEQ ID NO. 2); YARKARRQARR (SEQ ID NO. 3); YARAAARQARA (SEQ ID NO. 4); YARAARRAARR (SEQ ID NO. 5); YARAARRAARA (SEQ ID NO. 6); YARRRRRRRRR (SEQ ID NO. 7); or YAAARRRRRRR (SEQ ID NO. 8).

37. The fusion molecule of claims 1-36, wherein the protein transduction domain consists essentially of AGRKKRRQRRR (SEQ ID NO. 2), YARKARRQARR (SEQ ID NO. 3), YARAAARQARA (SEQ ID NO. 4), YARAARRAARR (SEQ ID NO. 5), YARAARRAARA (SEQ ID NO. 6), YARRRRRRRRR (SEQ ID NO. 7) or YAAARRRRRRR (SEQ ID NO. 8).

38. The fusion molecule of claims 1-37, wherein the protein transduction domain comprises at least one of the following sequences:

YARAAPRRR; YARAPRRARRR; YARAPRRPRRR; YARAAARPARA: YARAPARQARA; or YARAPARPARA.

39. The fusion molecule of any one of claims 1-38, wherein the protein transduction domain has a molecular weight of between from about 1 kDa to 50 kDa.

40. The fusion molecule of any one of claims 1-39, wherein the protein transduction domain increases transduction efficiency by between about 5 to about 10 fold up to about 100 fold as determined by a transduction efficiency assay.

41. The fusion molecule of any one of claim 2, wherein the anti-infective agent is one of an anti-viral drug, amebicide, anthelmintic, antibiotic, anti-fungal, anti-protozoan, or leprostatic drug.

42. The fusion molecule of claim 42, wherein the anti-viral agent is active against a retroviral infection.

43. The fusion molecule of claim 42, wherein the anti-viral agent is an AIDS chemotherapuetic agent.

44. The fusion molecule of claim 43, wherein the AIDS chemotherapuetic agent is a non-nucleoside reverse transcriptase inhibitor, nucleoside reverse transcriptase inhibitor, or a protease inhibitor.

45. A mammal comprising a fusion molecule comprising a covalently linked protein transduction domain (PTD) and linked molecule.

46. The mammal of claim 46, wherein the fusion molecule is a fusion protein and the linked molecule is a cytotoxic domain further comprising at least one pathogen-specific protease cleavage site.

47. The mammal of claim 46, wherein the fusion protein comprises covalently linked in sequence: 1) the transduction domain, 2) a first pathogen-specific protease cleavage site, and 3) a zymogen.

48. The mammal of claim 47, wherein the fusion protein comprises covalently linked in sequence: 1) the transduction domain, 2) a first pathogen-specific protease cleavage site, 3) a catalytic zymogen fragment, and 4) a first zymogen subunit.

49. The mammal of claim 48, wherein the fusion protein further comprises covalently linked to the C-terminal end of the first zymogen subunit, 5) a second pathogen-specific protease cleavage site, and 6) a second zymogen subunit.

50. The mammal of claim 49, wherein the zymogen is a caspase, thrombin, bacterial exotoxin, granzyme B, an invertebrate toxin, p53 or p19ARF.

51. The mammal of claim 50, wherein the caspase is selected from the group consisting of caspase-3 (CPP32 apopain, Yama), caspase-5 (ICE_{rel}-III, TY), caspase-4(ICE_{rel}-II TX, ICH-2), caspase-1 (ICE), caspase-7 (Mch3, ICE-LAP3, CMH-1), caspase-6 (Mch2), caspase-8 (MACH, FLICE, Mch5), caspase-10 (Mch4), caspase-2 (ICH-1), caspase-9 (ICH-LAP6, Mch6),) or a catalytically active zymogen.

52. The mammal of claim 51, wherein the caspase is caspase-3 (CPP32 apopain, Yama) or a catalytically active fragment thereof.

53. The mammal of claim 52, wherein the fusion protein further comprises covalently linked in sequence: 1) the transduction domain, 2) a pathogen-specific protease cleavage site, and 3) an enzyme.

54. The mammal of claim 53, wherein the enzyme is thymidine kinase, cytosine deaminase, or a catalytically active enzymatic fragment thereof.

55. The mammal of claim 54, wherein the enzyme is thymidine kinase.

56. The mammal of claims 45-55, wherein the pathogen-specific protease cleavage sites are selected from the group consisting of cytomegalovirus (CMV), herpes simplex virus type-1 (HSV-1); hepatitis virus type C (HCV); *P. falciparum*, HIV-1, HIV-2 and Kaposi's sarcoma-associated herpes virus (KSHV) protease cleavage sites.

57. The mammal of claim 45-56, wherein the fusion protein comprises covalently linked in sequence: 1) TAT or a protein transducing fragment thereof, 2) a caspase-3 prodomain, 3) a first HSV-1 protease cleavage site, 4) a caspase-3 large subunit, 5) a second HSV-1 protease cleavage site, and 5) a caspase-3 small subunit, wherein the large and small subunits are capable of dimerizing to form a cytotoxin.

58. The mammal of claims 45-57, wherein the protein transduction domain is TAT, Antennapedia homeodomain, HSV VP22 or a fragment thereof.

59. The mammal of claims 45-58, wherein the protein transduction domain is a synthetic peptide or other transducing protein.

60. The mammal of claims 45-59 in which the fusion protein further comprises a protein purification or identification tag.

61. The mammal of claim 60, wherein the protein purification or identical tag is a polyhistidine, EE, MYC, HA sequence, or other peptide capable of being bound by an antibody.

62. The mammal of any one of claims 40-61, wherein the protein transduction domain comprises at least a peptide represented by the following formula: B_1 -X_1-X_2-X_3-B_2-X_4-X_5-B_3, wherein B_1, B_2, and B_3 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3 , X_4 and X_5 are each independently an alpha-helix enhancing amino acid the same or different.

63. The mammal of any one of claims 40-62, wherein the protein transduction domain represented by the formula B_1 -X_1-X_2-X_3-B_2-X_4-X_5-B_3, wherein B_1, B_2, and B_3 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3 , X_4 and X_5 are each independently an alpha-helix enhancing amino acid the same or different.

64. The mammal of claim 62 or 63, wherein at least one of the basic amino acids is arginine.

65. The mammal of claim 62 or 63, wherein at least one of the alpha-helix enhancing amino acids is alanine.

66. The mammal of claim 62 or 63, wherein at least one of the alpha-helix enhancing amino acids is alanine and the basic amino acids are arginine substantially aligned along at least one face of the peptide.

67. The mammal of any one of claims 40-66, wherein the protein transduction domain comprises at least a peptide represented by the following formula: B_1 -X_1-X_2-B_2 -B_3 -X_3-X_4-B_4; wherein B_1, B_2, B_3, and B_4 are each independently a basic amino acid, the same or different; and X_1, X_2, X_3 , and X_4 are each independently an alpha-helix enhancing amino acid the same or different.

68. The mammal of any one of claims 40-67, wherein the protein transduction domain comprises the following formula: B_1 -X_1-X_2-B_2 -B_3 -X_3-X_4-B_4; wherein B_1, B_2, B_3, and B_4 are each independently a

basic amino acid, the same or different; and X_1, X_2, X_3, and X_4 are each independently an alpha-helix enhancing amino acid the same or different.

69. The mammal of claim 67 or 68, wherein at least one of the basic amino acids is arginine.

70. The mammal of claim 67 or 68, wherein at least one of the alpha-helix enhancing amino acids is alanine.

71. The mammal of claim 67 or 68, wherein at least one of the alpha-helix enhancing amino acids is alanine and the basic amino acids are arginine substantially aligned along at least one face of the peptide.

72. The mammal of any one of claims 40-71, wherein the protein tranduction domain comprises at least the following sequences: AGRKKRRQRRR (SEQ ID NO. 2); YARKARRQARR (SEQ ID NO. 3); YARAAARQARA (SEQ ID NO. 4); YARAARRAARR (SEQ ID NO. 5); YARAARRAARA (SEQ ID NO. 6); YARRRRRRRRR (SEQ ID NO. 7); or YAAARRRRRRR (SEQ ID NO. 8).

73. The mammal of any one of claims 40-72, wherein the protein transduction domain consists essentially of AGRKKRRQRRR (SEQ ID NO. 2), YARKARRQARR (SEQ ID NO. 3), YARAAARQARA (SEQ ID NO. 4), YARAARRAARR (SEQ ID NO. 5), YARAARRAARA (SEQ ID NO. 6), YARRRRRRRRR (SEQ ID NO. 7) or YAAARRRRRRR (SEQ ID NO. 8).

74. The mammal of any one of claims 40-73, wherein the protein transduction comprises at least one of the following sequences: YARAAPRRR; YARAPRRARRR; YARAPRRPRRR; YARAAARPARA: YARAPARQARA; or YARAPARPARA.

75. The mammal of any one of claims 40-74, wherein the protein transduction domain has a molecular weight of between from about 1 kDa to 50 kDa.

76. The mammal of claims 40-75. wherein the protein transduction domain increases transduction efficiency by between about 5 to about 10 fold up to about 100 fold as determined by a transduction efficiency assay.

77. The mammal of claim 76, wherein increase in transduction efficiency is expressed as an increase in intracellular concentration of the transduction domain.

78. The mammal of any one of claims 40-77, wherein the fusion protein comprises covalently linked in sequence: 1) a transduction domain, 2) a first zymogen subunit, 3) a protease cleavage site, and 4) a second zymogen subunit.

79. The mammal of claim 78, wherein the transduction domain is TAT, the first zymogen subunit is p5 Bid, the protease cleavage site is an HIV protease cleavage site and the second zymogen subunit is p15 Bid.

80. The mammal of any one of claims 40-79, wherein the fusion protein comprises covalently linked in sequence: 1) a transduction domain, 2) a first protease cleavage site, 3) first zymogen subunit, 3) a second protease cleavage site, and 4) a second zymogen subunit.

81. The mammal system of claim 80, wherein the transduction domain is TAT, the first protease cleavage site is an HIV p7-p1 protease cleavage site, the first zymogen subunit is p17 caspase-3, the second protease cleavage site is an HIV p17-p24 protease cleavage site, and the second zymogen subunit is p12 caspase-3.

82. The mammal of claim 81, wherein the p17 caspase-3 comprises a Cys^{163} to Met^{163} mutation.

83. The mammal of any one of claims 40-82, wherein the mammal is a primate, rodent or a rabbit.

84. The mammal of claim 83, wherein the rodent is a mouse or a rat.

85. The mammal of claims 40-84, wherein the half-life (LD_{50}) of the fusion molecule in vivo or in vitro is from between about 1 hour to 72 hours.

86. The mammal of claim 85, wherein the half-life (LD_{50}) of the fusion molecule in vivo is from between about 6 hours to 24 hours.

87. The mammal of claim 83, wherein the rodent is a mouse.

88. The mammal of any one of claims 40-87, wherein the mammal comprises a transduced fusion molecule expressed in a cell, group of cells including tissue, organ, group of organs, physiological system including the circulatory, lymphatic and immune system, up to essentially the entire mammal.

89. A kit comprising:

a mammal such as a mouse, rodent, or primate used for research which kit comprises means for transducing at least one fusion molecule into the mammal, the means comprising at least one fusion molecules of claims 1-39 or means for making that fusion molecule in accord with directions provided in the kit.

90. The kit of claim 89 further comprising a syringe or related implementation for introducing the fusion molecule into the mammal such as a rodent or mouse and means for detecting that fusion molecule,

which means will include at least one antibody for detecting the fusion molecule.

91. A method of treating or preventing a medical condition in a mammal, the method comprising administering to the mammal a therapeutically effective amount of the fusion molecule of any one of claims 1-39 in an amount sufficient to treat or prevent that condition in the mammal.

92. The method of claim 91, wherein the mammal is a rodent, rabbit or primate acceptable for research purposes.

93. The method of claim 92, wherein the rodent is a rat or mouse and the primate is a chimpanzee or monkey.

94. A method for testing a fusion molecule for therapeutic capacity to inhibit a medical condition in a mammal, the method comprising administering the fusion molecule of any one of claims 1-39 to the mammal in an amount sufficient to treat or prevent the medical condition in the mammal.

95. The method of claim 94, wherein the mammal is a rodent, rabbit or primate.

96. The method of claim 96, wherein the rodent is a rat or mouse and the primate is a chimpanzee or monkey.

97. The method of claims 91-97 further comprising transducing the fusion molecule into cells, tissues, organs, groups of organs, physiological system up to essentially the entire mammal, cleaving the fusion molecule; and releasing the previously linked molecule inside cells.

98. The method of any one of claims 91-97, wherein the mammal is a primate, rodent, or a rabbit.

99. The method of claims 91-97, wherein the primate is a human patient and the medical condition is at least one of Alzheimer's disease, pre-senile dementia, age-related memory loss, Huntington's disease, epilepsy including seizures, a physchiatric disorder including compulsive behaviors, Parkinson's disease, Huntington's disease, or Lou Gehring's disease.

100. The method of any one of claims 91-99, wherein the fusion molecule comprises a prodrug.

101. A method of treating or preventing infection by an infectious agent, the method comprising administering to a mammal a therapuetically effective amount of any one of the fusion molecules of claims 1-39.

101. The method of claim 101, wherein the infectious agent is a pathogenic
virus, yeast, bacterium, nematode, fungus, helminth, prion, or protozoan.

102. The fusion molecule of any one of claims 1-40, wherein the fusion molecule has a molecular weight of at least about 100 kDa.

103. The fusion molecule of claim 102, wherein the linked molecule is a biologically active protein.

104. The fusion molecule of claim 103, wherein the fusion molecule is capable of crossing the blood:brain barrier.

105. The fusion molecule of claim 104, wherein the linked molecule retains its biological activity once transduced into the brain cells.

106. A mammal adapted for experimental use and comprising at least one transduction molecule transduced into essentially all of the cells in the mammal.

107. The mammal of claim 106, wherein the mammal is a rabbit, mouse, rat, or a primate selected from the group consisting of chimpanzee, orangutan, baboon, or member of the monkey family.

108. A method for discovering a candidate molecule with therapuetic activity, the method comprising:

a) making a transduction molecule that includes at least one of the molecules covalently linked to a suitable transducing protein,
b) transducing the transduction molecule into suitable cells,
c) screening the cells for presence or absence of the molecule; and
d) detecting the candidate molecule with the therapuetic activity.

109. The method of claim 108, wherein the screening step further comprises identifying a phenotype indicative of the cell having the molecule tranduced therein.

110. The method of claim 108 or 109, wherein the cells are cultured animal cells employed in a convential drug screen or the cells are present as tissue or an organ.

111. The method of any one of claims 108-110, wherein the screening step further comprises testing the cells for presence of absence of candidate molecule in a suitable animal model.

112. A molecule having therapuetic activity identified by any one of the methods of claims 108-111.

113. A method for killing or damaging microbes having significant resistance to a therapuetic molecule, the method comprising:

a) making a transduction molecule that includes at least one of the molecules covalently linked to a suitable transducing protein,

b) transducing the transduction molecule into microbes under conditions that kill or damage the microbes; and

c) killing or damaging the molecules with the transduction molecule.

114. The method of claim 113, wherein the therapuetic molecule is an antibiotic or a sulfa drug (sulfonamide)

115. The method of claim 114, wherein the antibiotic is penicillin, ampicillin, bacitracin, erythromycin, amoxicillin, cephalosporin; or a derivative thereof.

116. The method of claim 114, wherein the sulfa drug is trimethoprim.

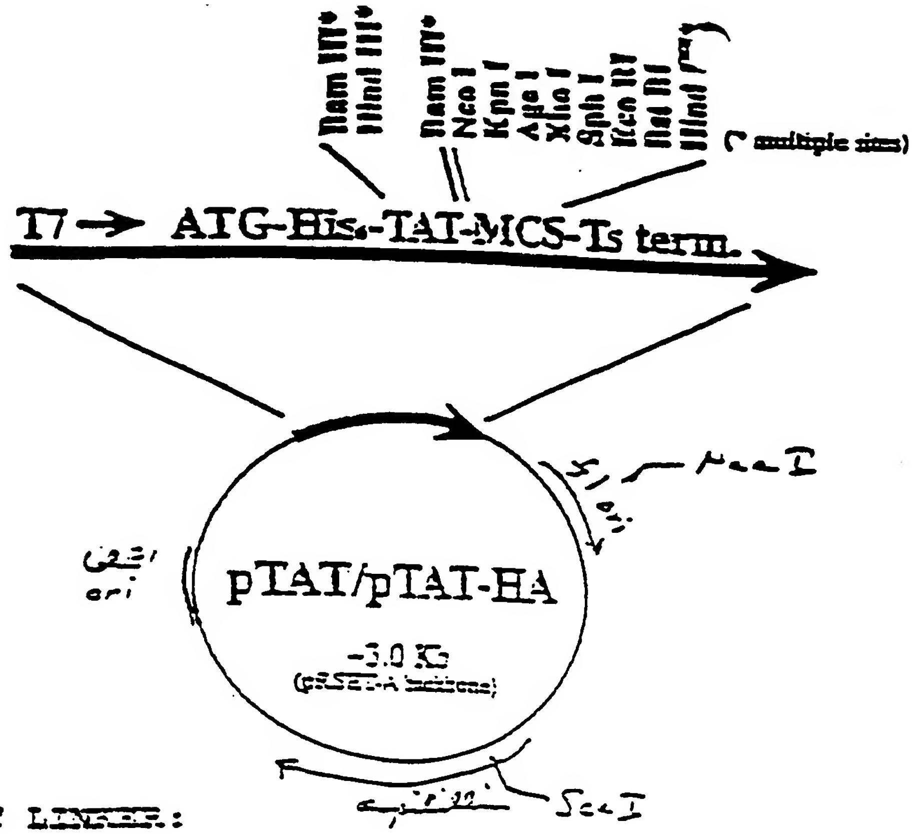


FIG. 1

pTAT LINKER:

```
BamHI HindIII                          TAT DOMAIN
GGA TCC AAG CTT GGC TAC GGC CGC AAG AAA CGC CGC CAG CGC CGC CGC GGT
 G   S   K   L   G   Y   G   R   K   K   R   R   Q   R   R   R   G

BamHI     NcoI       KpnI AgeI   XhoI     SphI        Eco BstBI HindIII
GGA TCC ACC ATG GCT GGT ACC GGT CTC GAG GTG CAT GCG GTG AAC TCG AAG CTT
 G   S   T   M   A   G   T   G   L   E   V   H   A   V   N   S   K   L
```

FIG. 2

-followed by 20 amino acids to TAA Ts termination codon.

pTAT-HA LINKER:

The HA tag, flanked by glycine residues, was inserted into the NcoI site of pTAT. The N' NcoI site has been inactivated.

```
original                                                    NEW
NcoI-(inactive)               AatII                         NcoI
CC ATG TCC GGC TAT CCA TAT GAC GTT CCA GAC TAT GCT GGC TCC ATG GGC ...
    M   S   G   Y   P   Y   D   V   P   D   Y   A   G   S   M   G
```

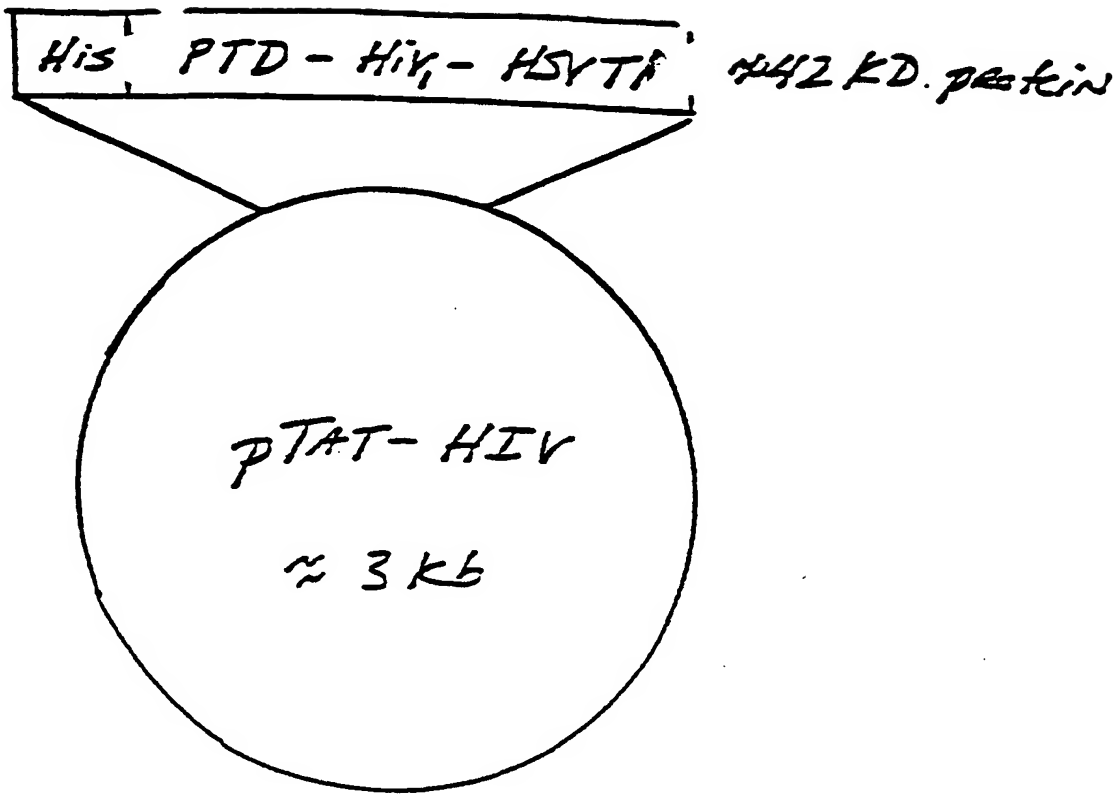

His
PTD - Hiv1 - HSVTK
~42 KD. protein
pTAT - HIV
~ 3 Kb

Fig. 3A

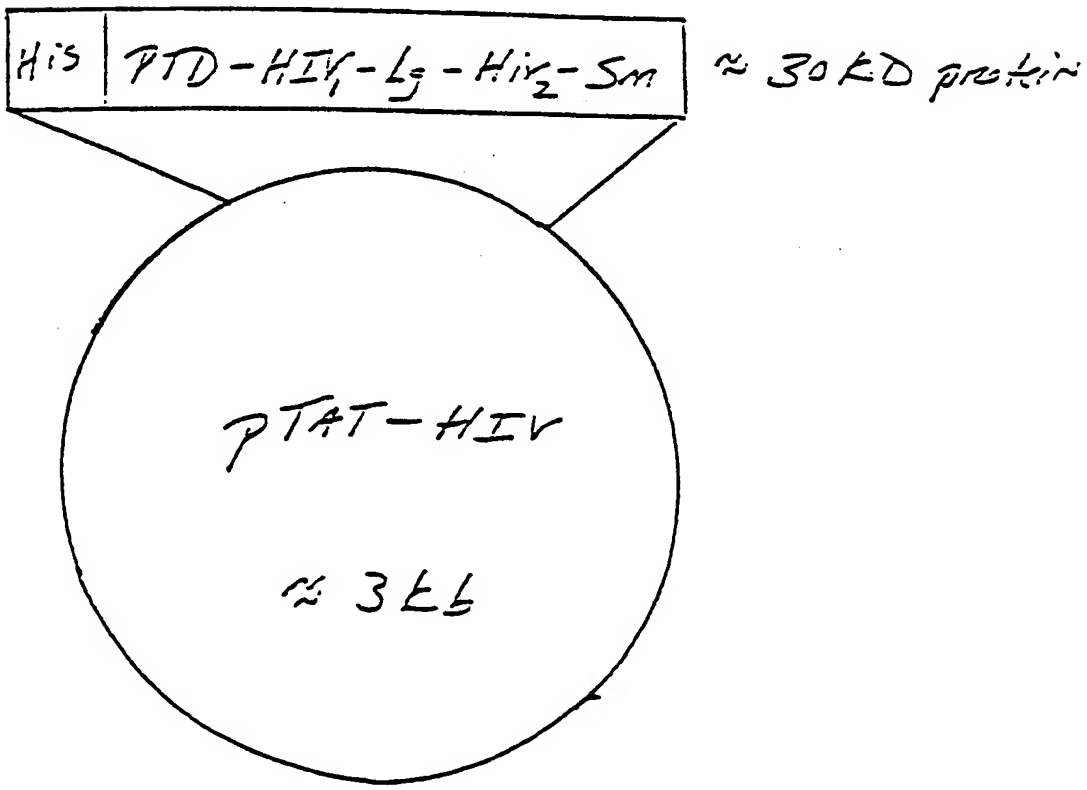

His
PTD - HIV1 - Lg - Hiv2 - Sm
~ 30 KD protein
pTAT - HIV
~ 3 Kb

Fig. 3B

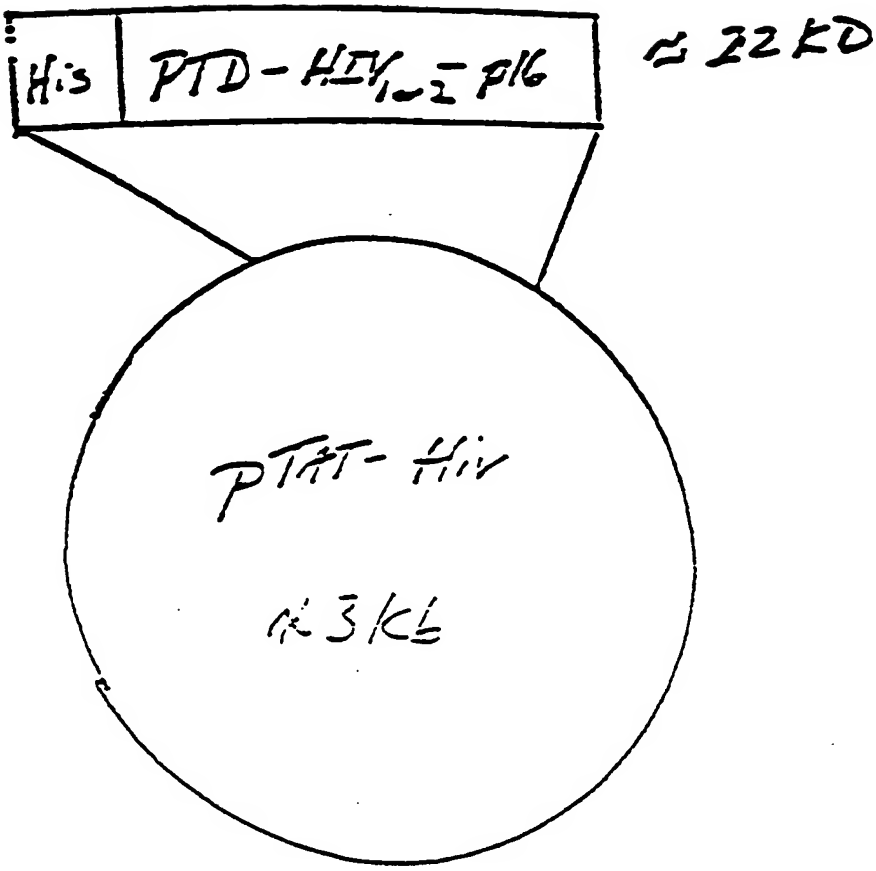

His
PTD-HIV1-2 p16
≈ 22 KD
pTAT-Hiv
≈ 3 KD

Fig. 3C

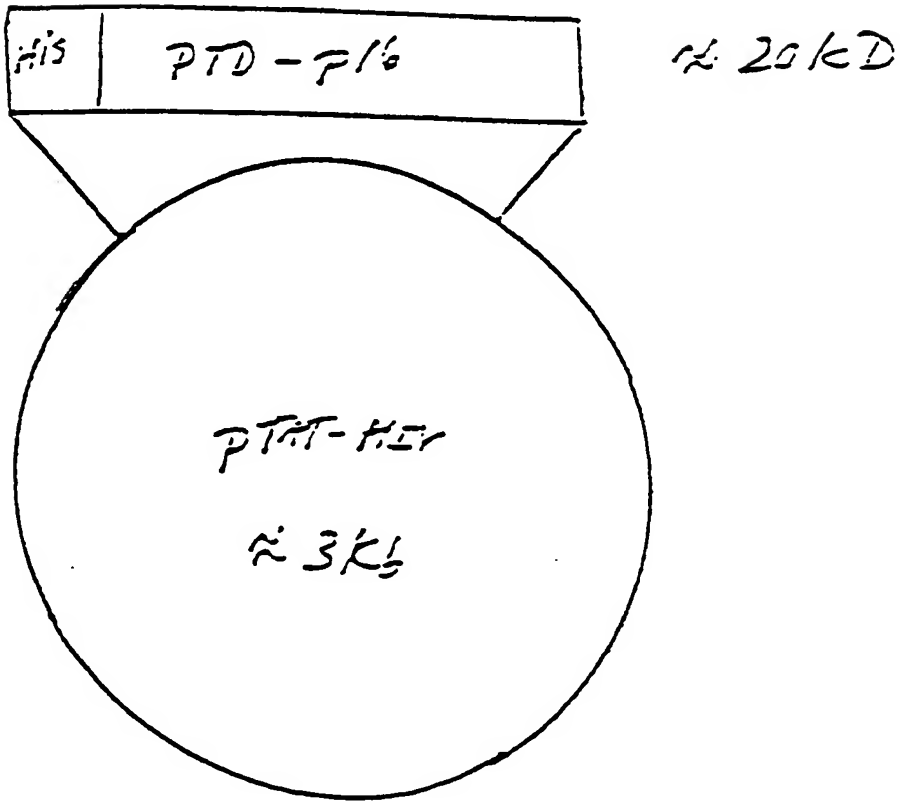

His
PTD-p16
≈ 20 KD
pTAT-HIV
≈ 3 KD

Fig. 3D

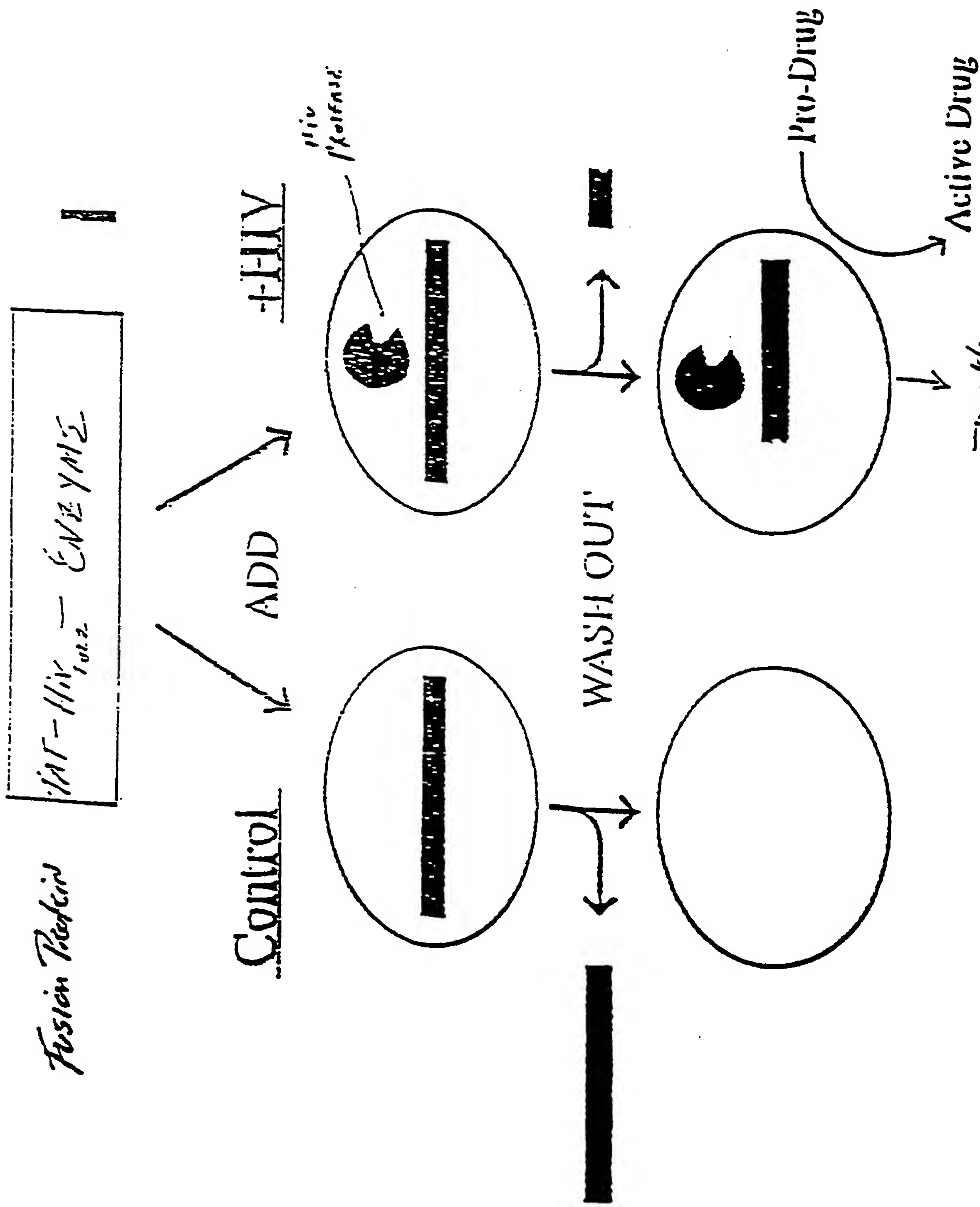

Fusion Protein
ENZYME
Control
+HIV
ADD
WASH OUT
Pro-Drug
Active Drug
Death

Fig. 1

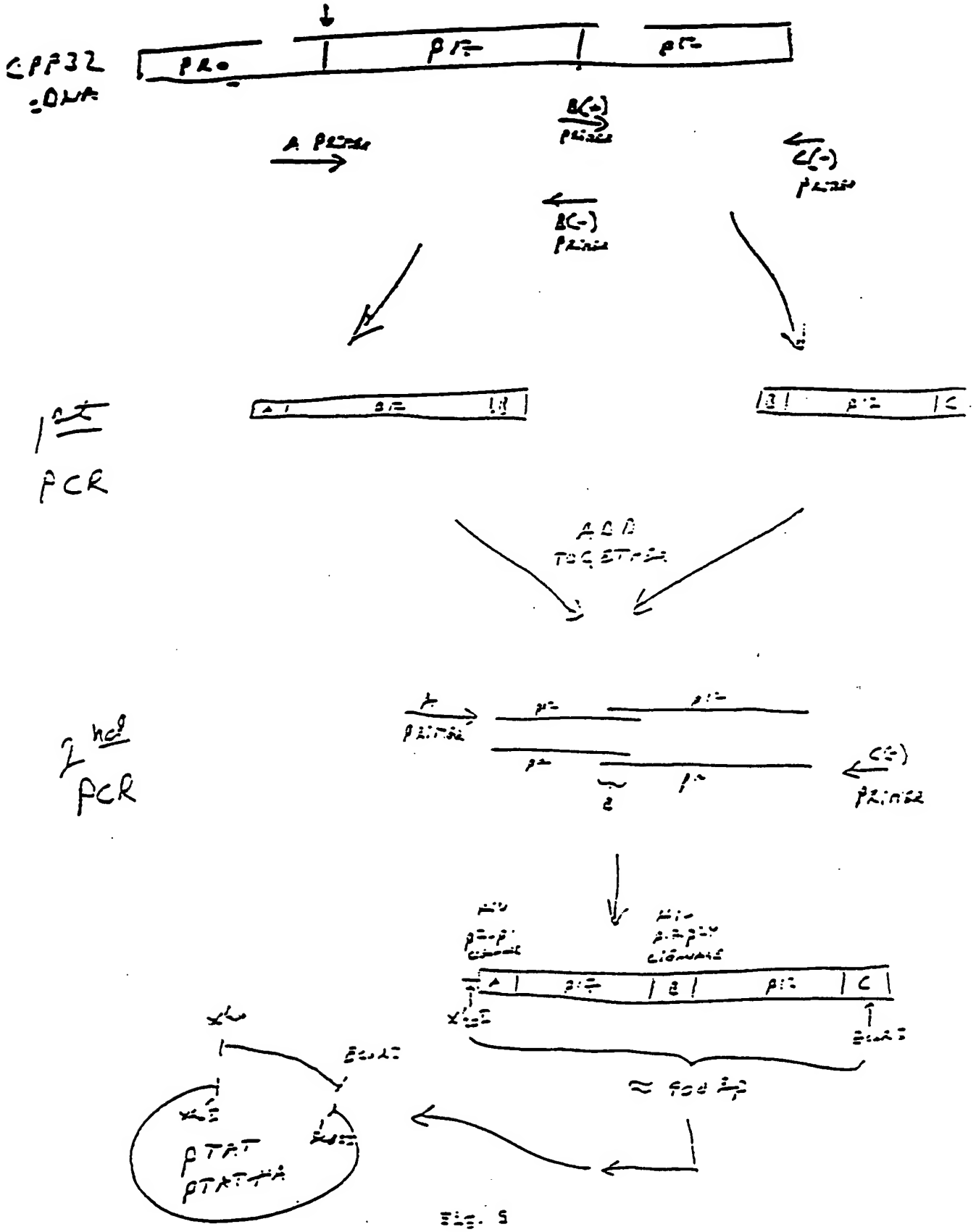


Fig. 5

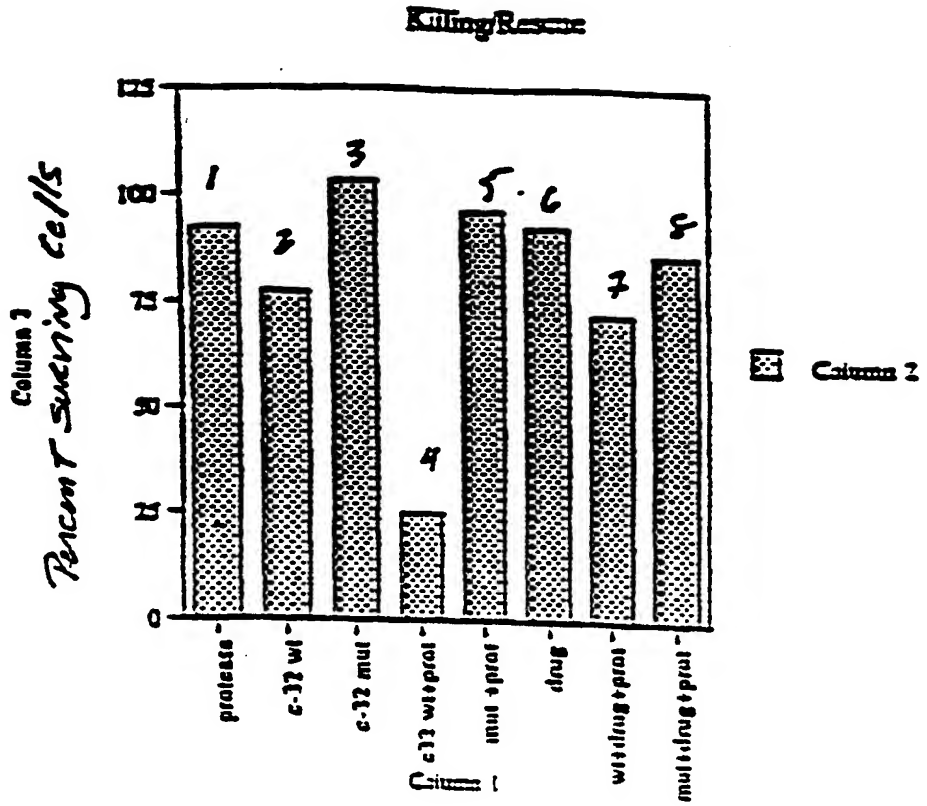
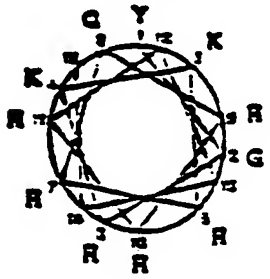


Fig. 6A

	1	2
	Column 1	Column 2
1	proteasa	92
2	c-32 wt	77
3	c-32 mut	103
4	c32 wt+prot	25
5	mut +prot	96
6	drug	92
7	wt-drug-pro	72
8	mut-drug-pr	85

Fig. 6B

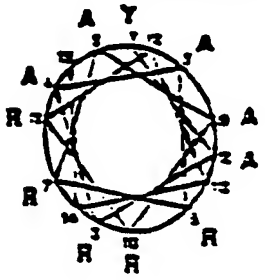
TAT (47-57)
YGRKKRRQRRR



Relative Intracellular Concentration

1x

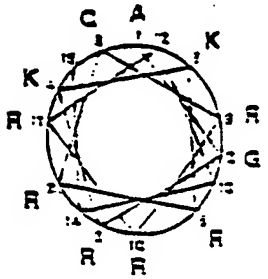
SFD#5
YARAARRAARR



Relative Intracellular Concentration

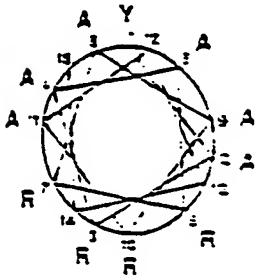
10x

SFD#2
AGRKKRRQRRR



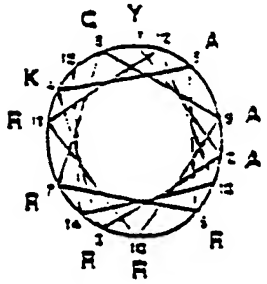
1x

SFD#6
YARAARRAARA



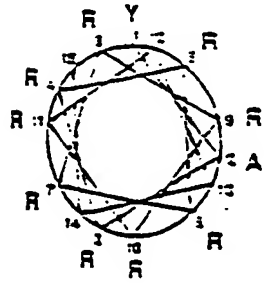
5x

SFD#3
YARKARRQARR



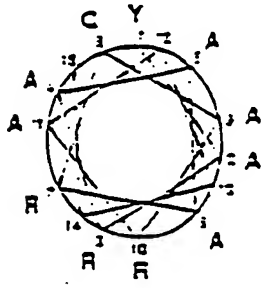
10x

SFD#7
YARRRRRRRRR



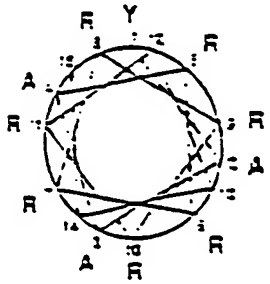
5x

SFD#4
YARAAARQARA



50X

SFD#8
YAAARRRRRRR



5x

FIGURE 7

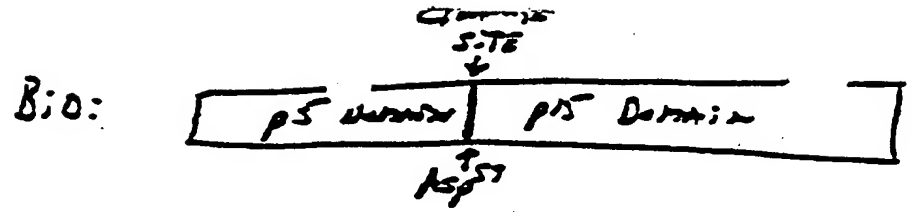
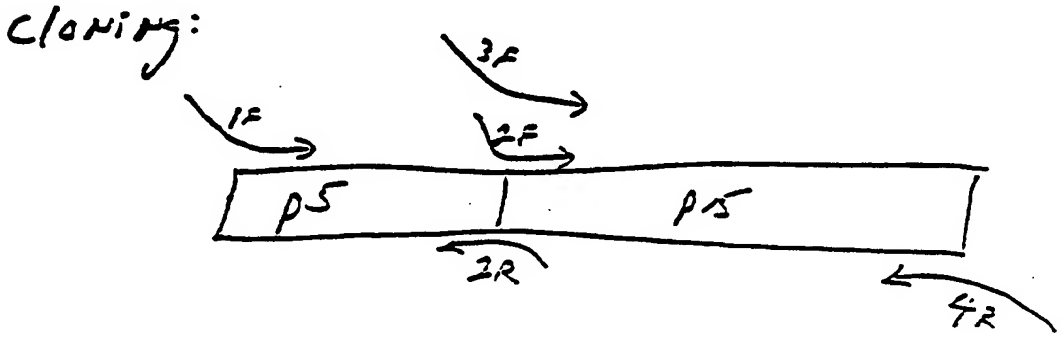
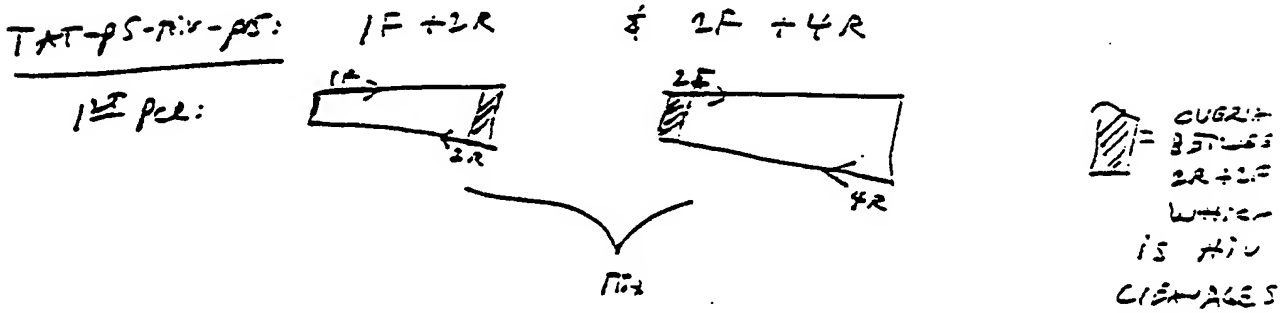


Figure 8 A





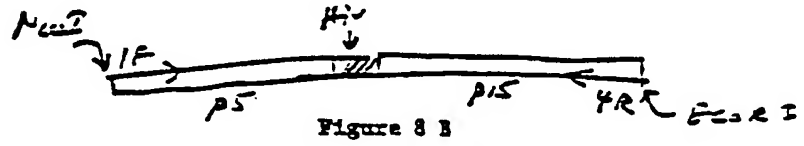


Figure 8 B

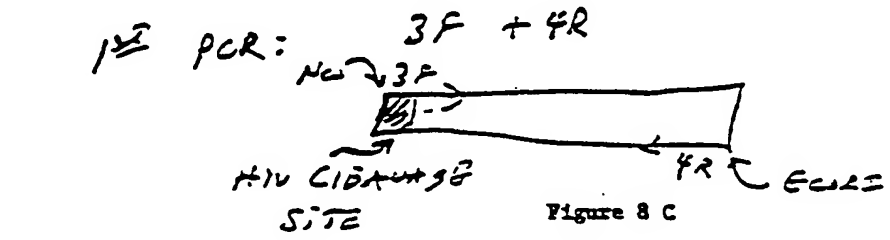


Figure 8 C

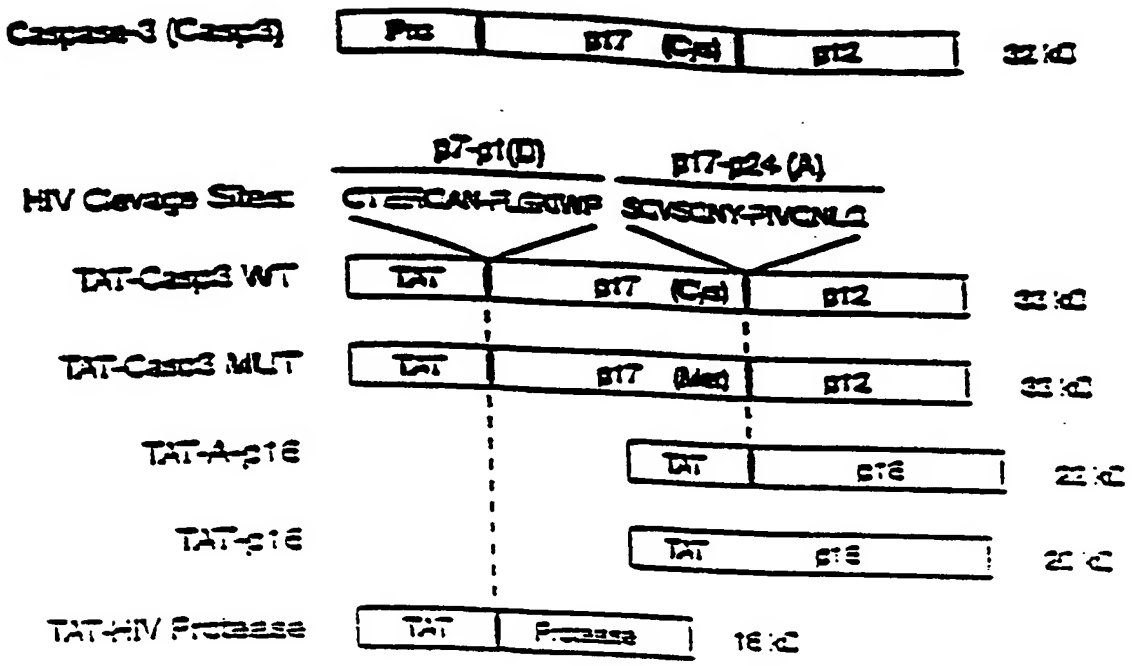


Figure 9 A

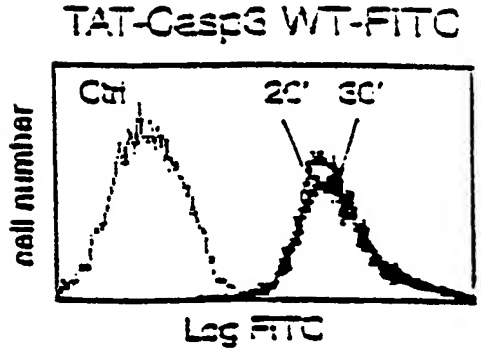


Figure 9 B

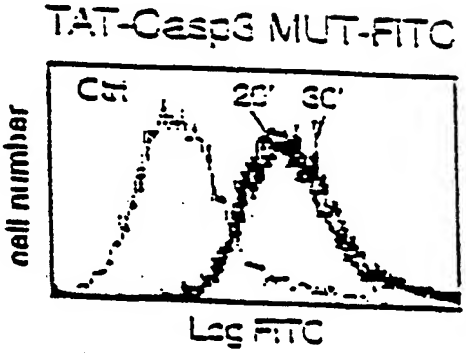


Figure 9 C

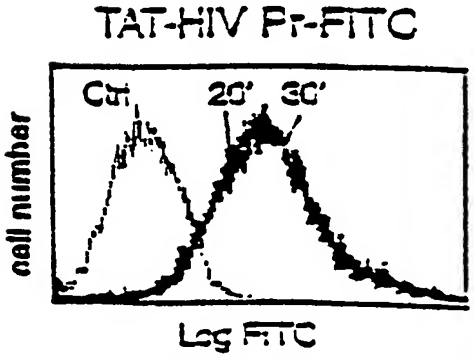


Figure 9 D

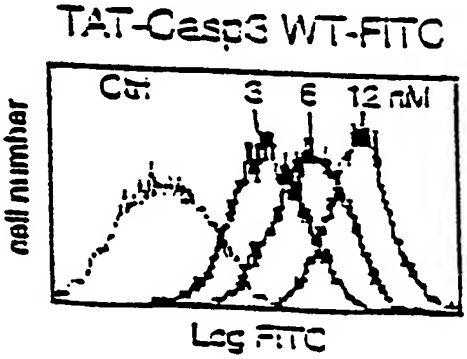


Figure 9 E

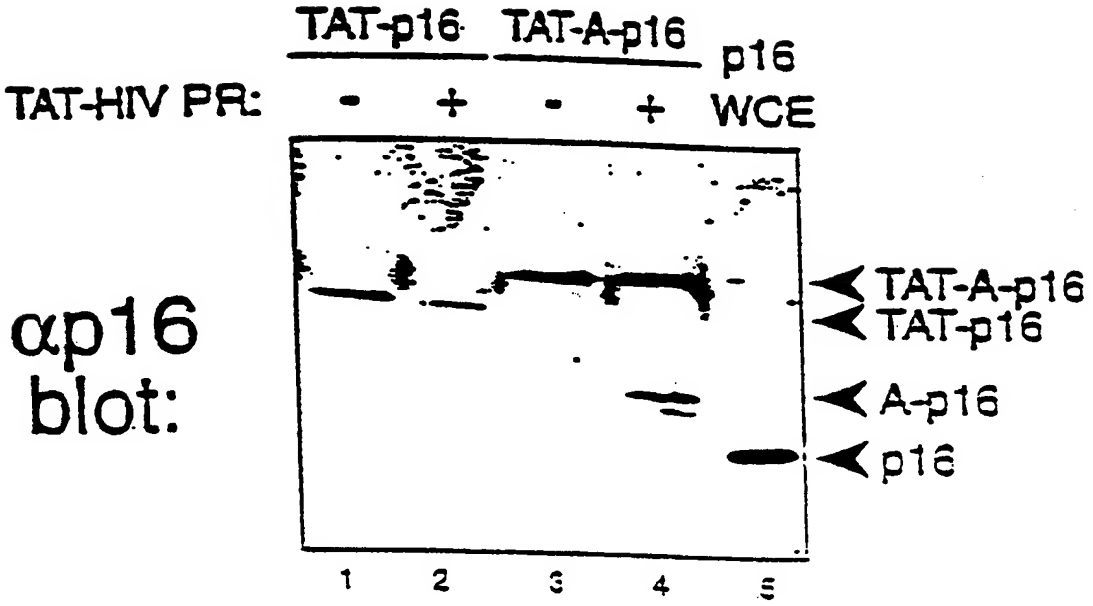


Figure 10 A

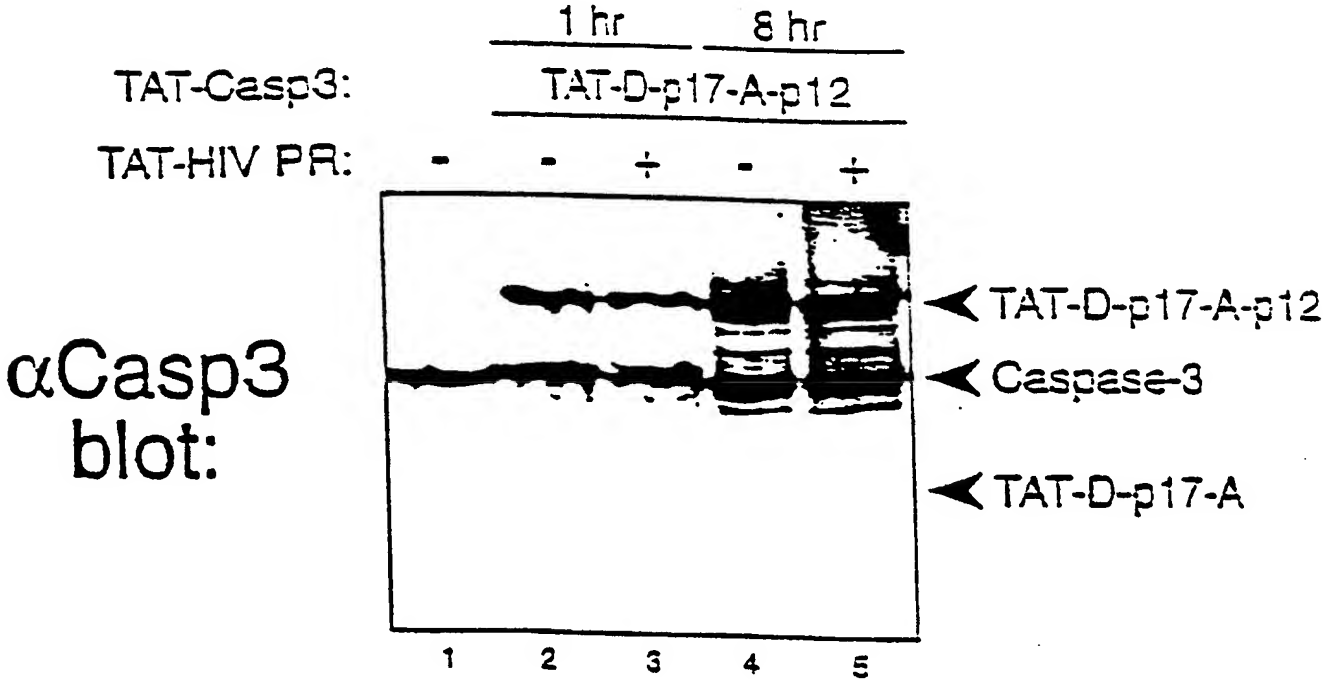


Figure 10 B

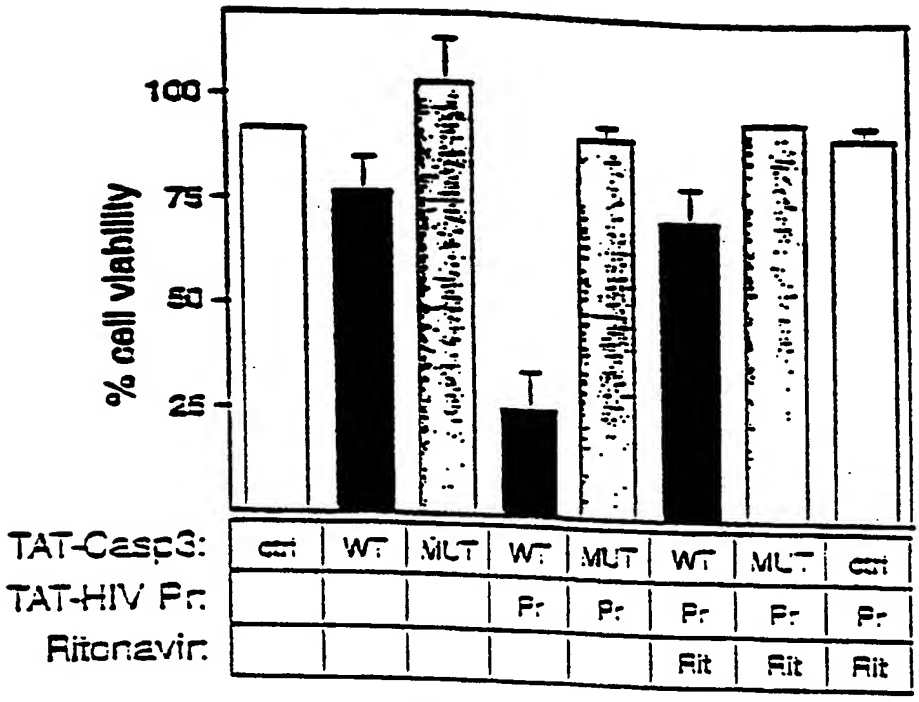


Figure 11 A

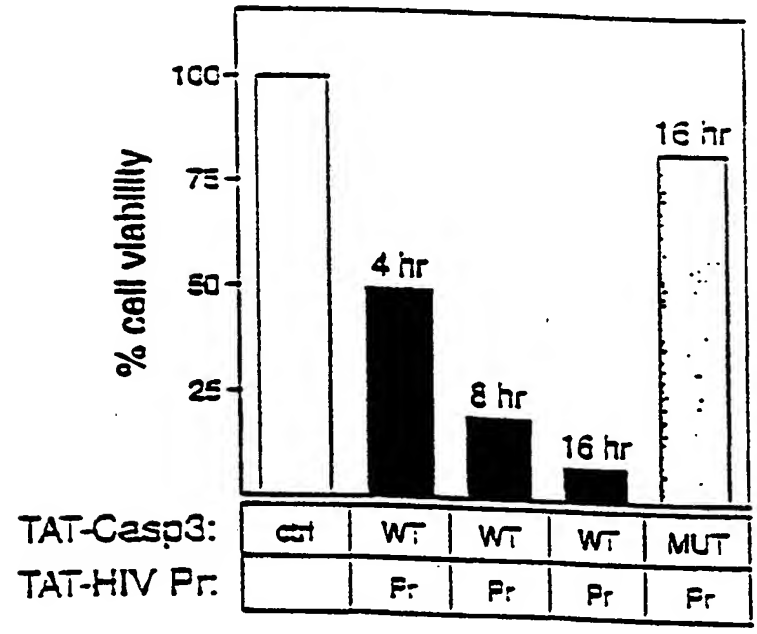


Figure 11 B

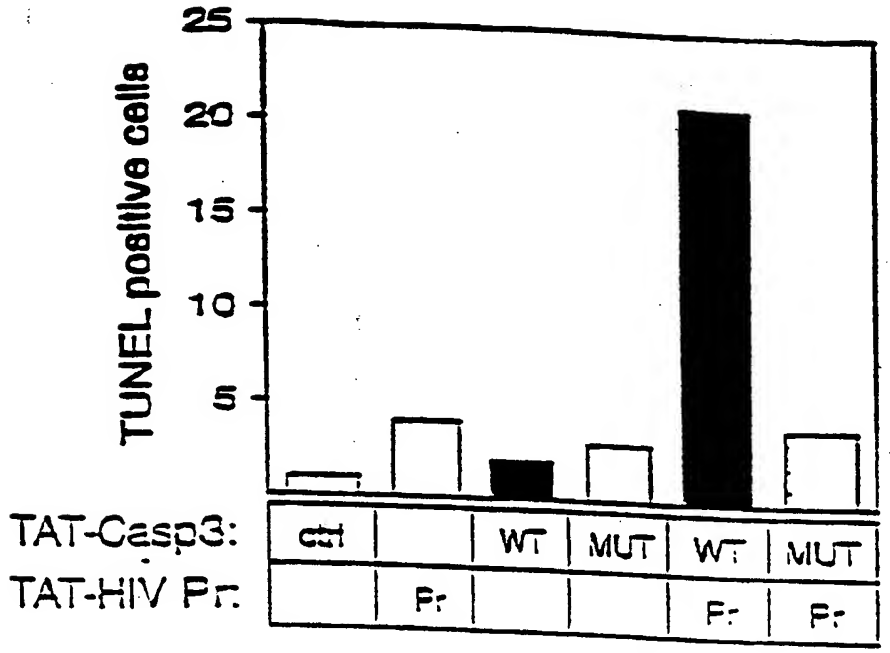


Figure 12 A

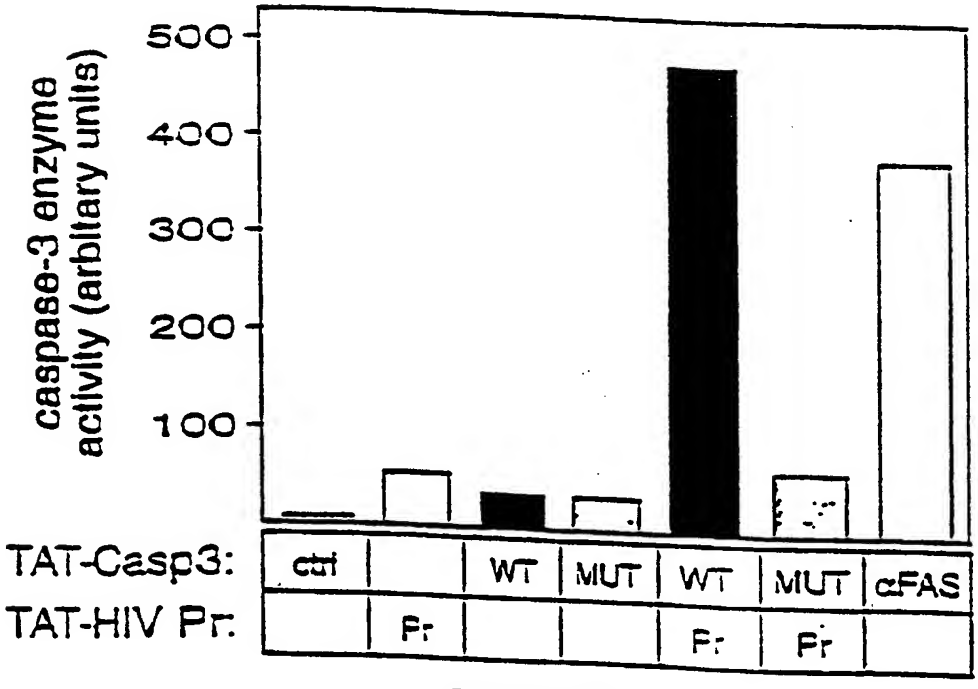


Figure 12 B

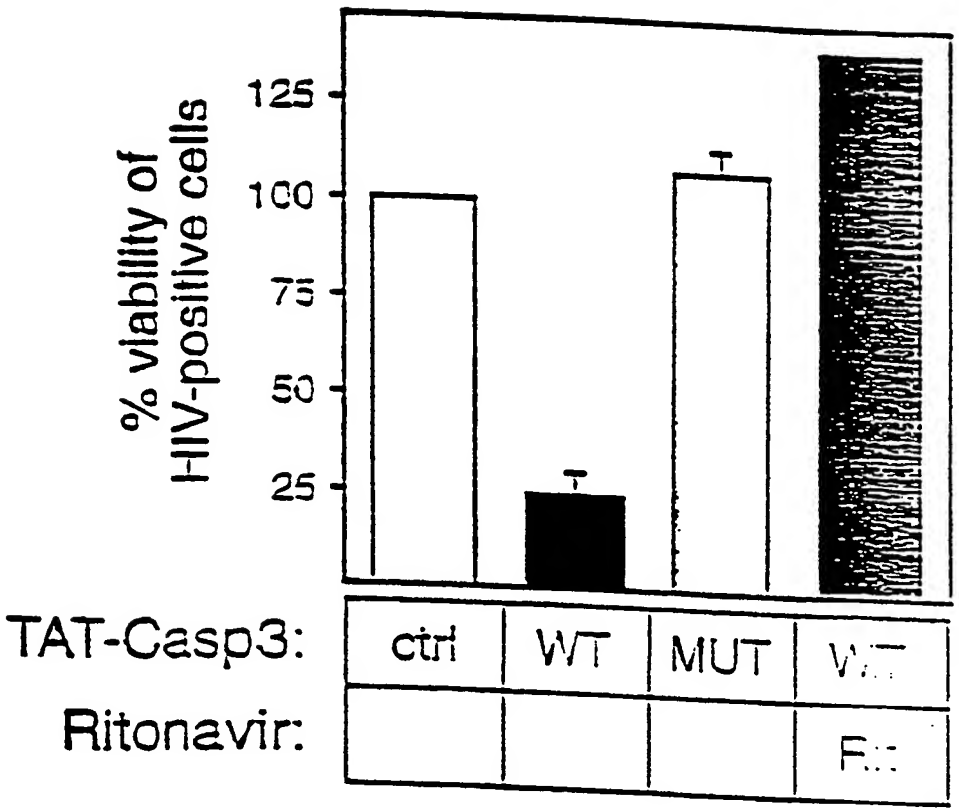
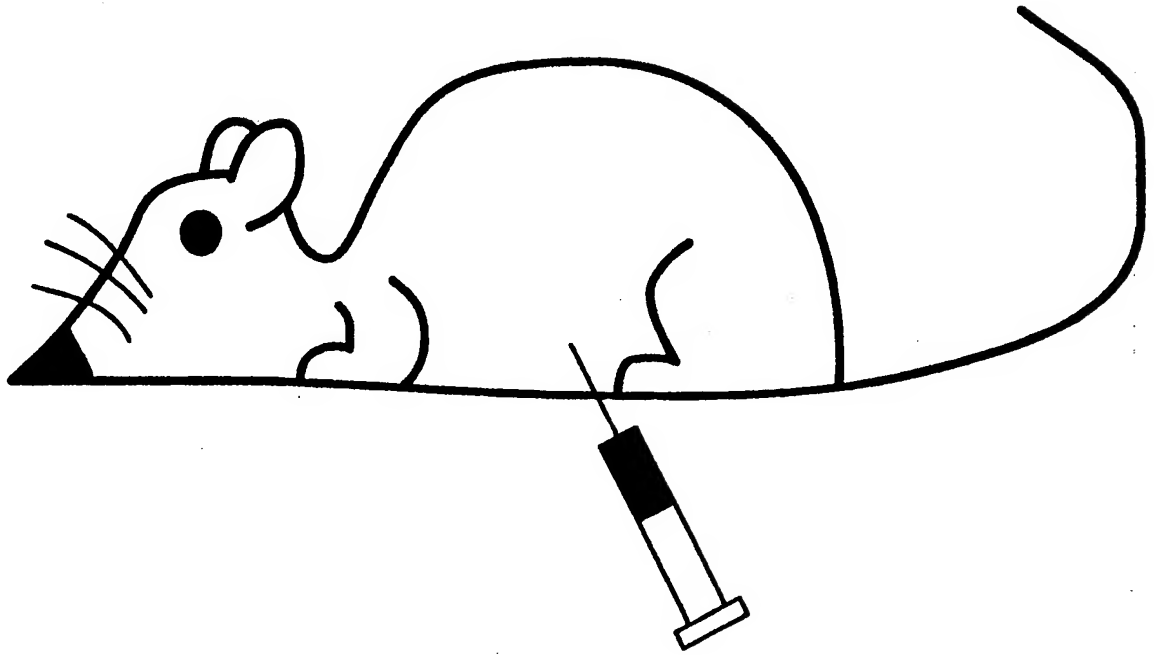

% viability of HIV-positive cells
125
100
75
50
25
TAT-Casp3:
ctrl
WT
MUT
WT
Ritonavir:
R

Figure 13

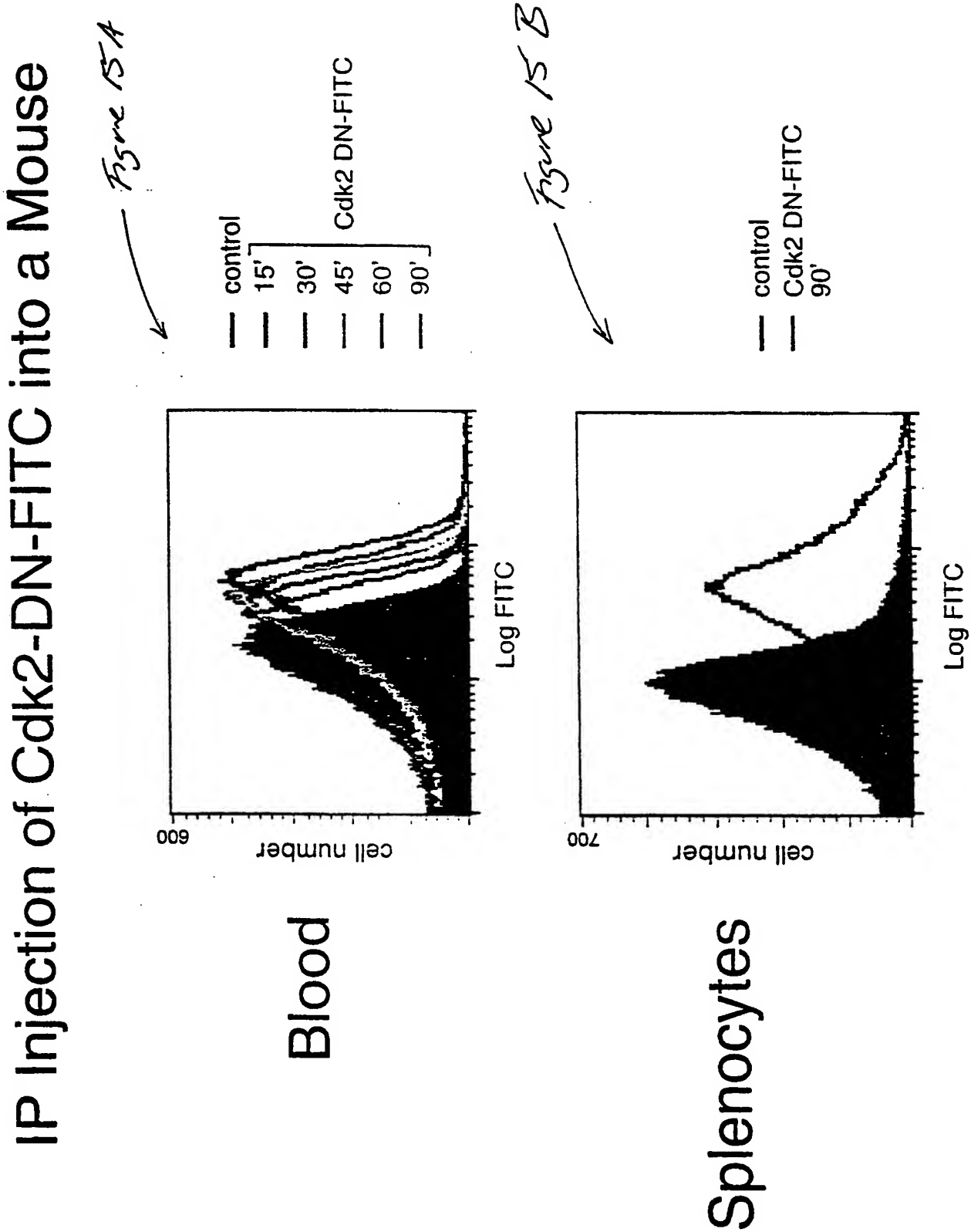


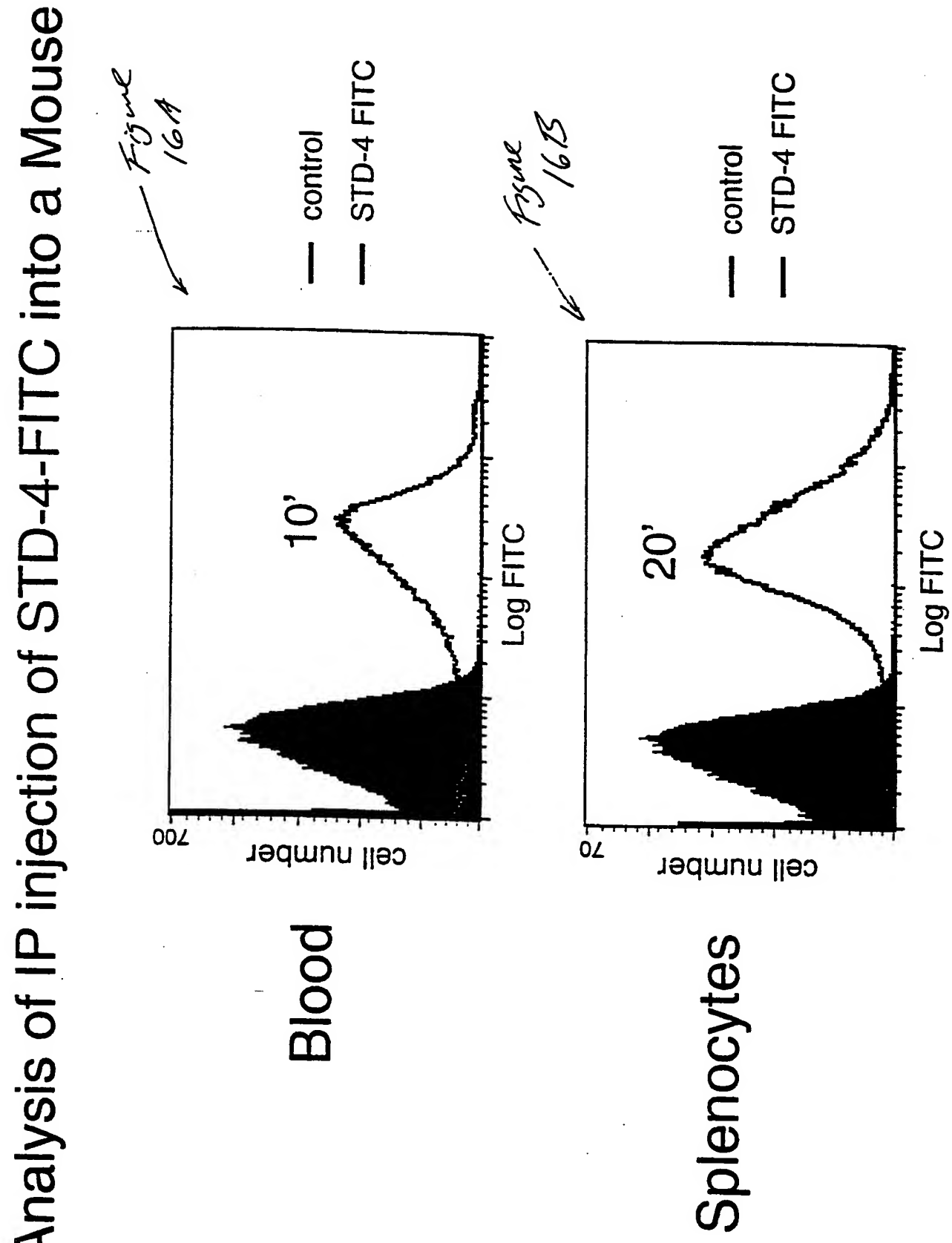
1. I.P. injection of **FITC** labeled transducible protein

2. Sample blood by Flow Cytometry over time

3. Perform splenectomy and analyze Splenocytes

Figure 14


IP Injection of Cdk2-DN-FITC into a Mouse
Figure 15A
control
15'
30'
45'
60'
90'
Cdk2 DN-FITC
Blood
cell number
600
Log FITC
Figure 15B
control
Cdk2 DN-FITC
90'
Splenocytes
cell number
700
Log FITC


Analysis of IP injection of STD-4-FITC into a Mouse
Figure 16A
control
STD-4 FITC
10'
cell number
700
Log FITC
Blood
Figure 16B
control
STD-4 FITC
20'
cell number
70
Log FITC
Splenocytes

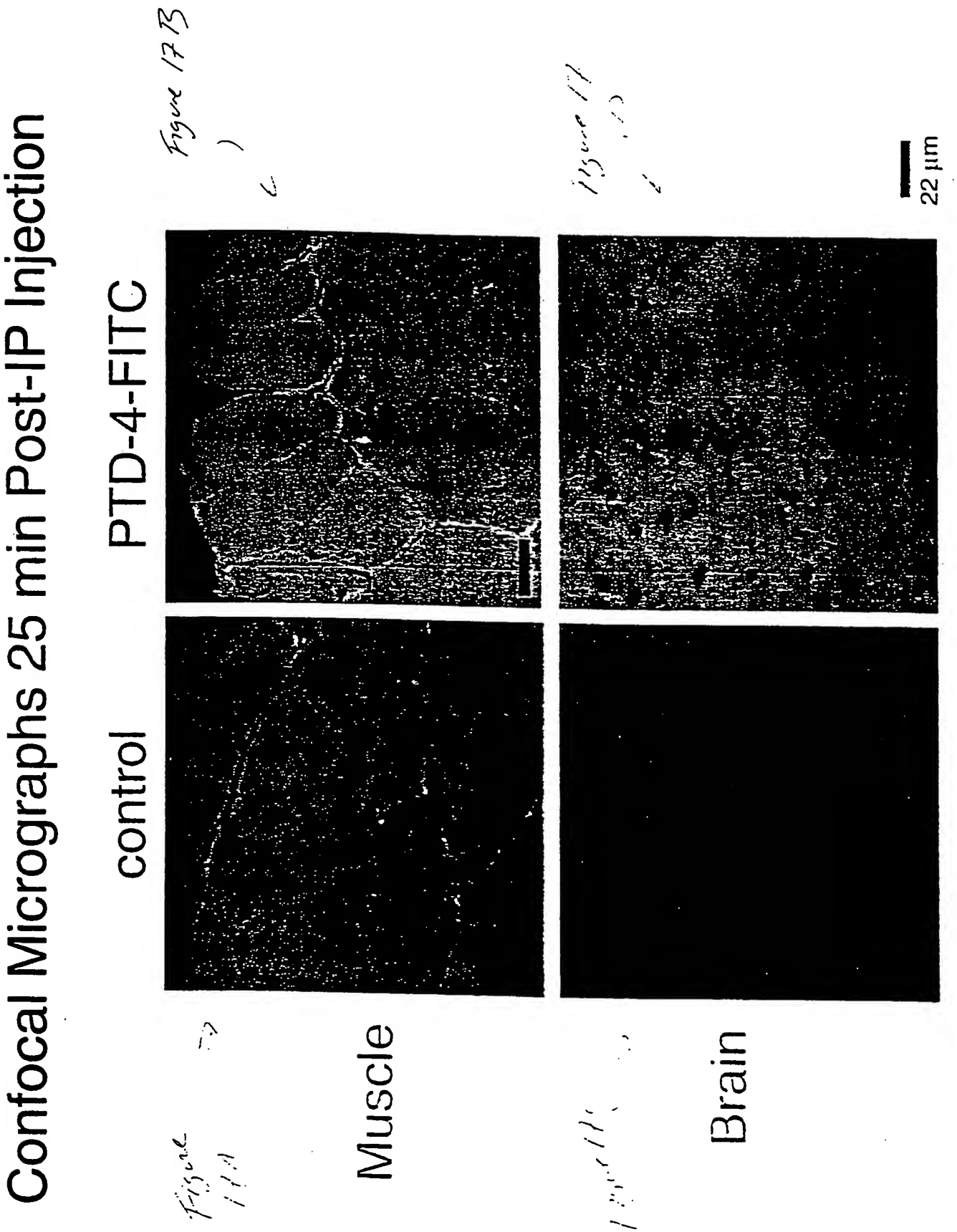
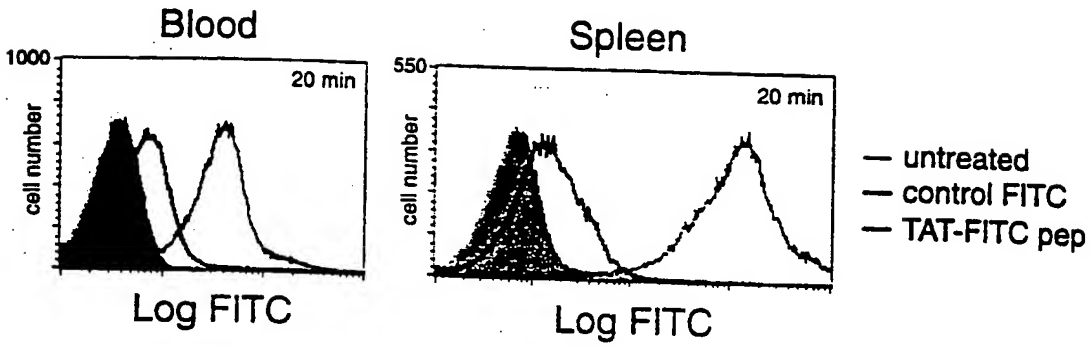

Confocal Micrographs 25 min Post-IP Injection
control
PTD-4-FITC
Muscle
Brain
22 µm

Fig. 18A

Fig. 18B



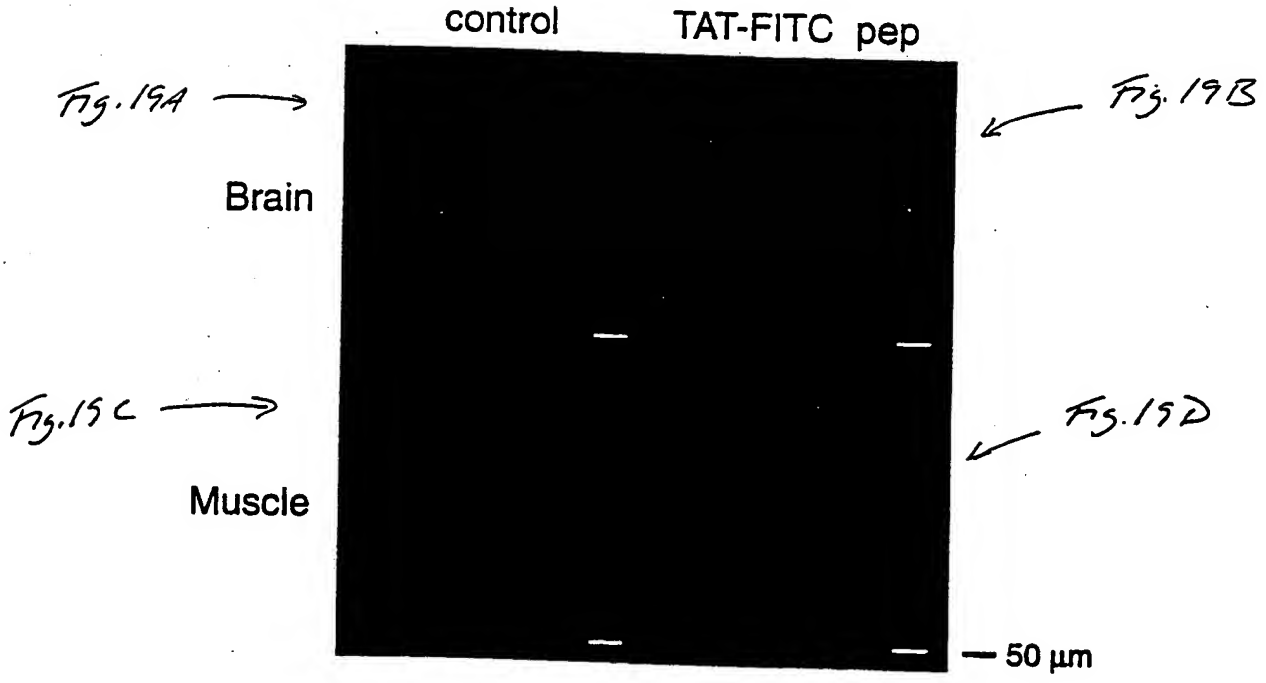


Fig. 20A

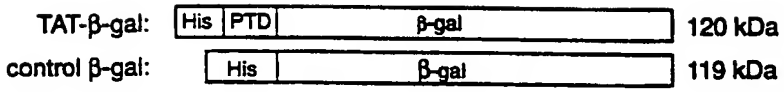


Fig. 20B

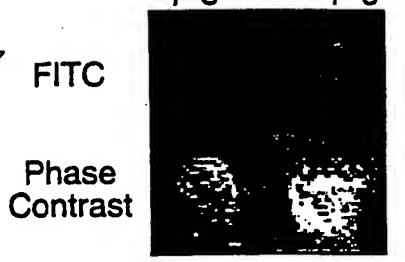
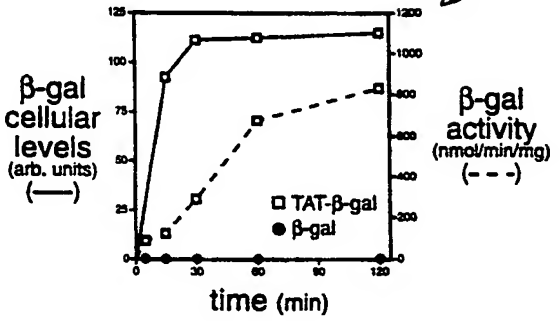


Fig. 20C



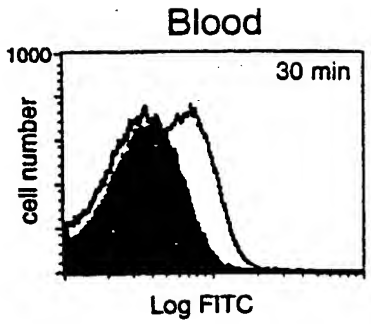


Fig. 20D

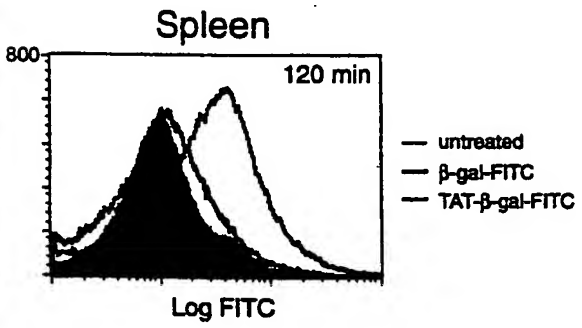


Fig. 20E

Fig. 21A Fig. 21B

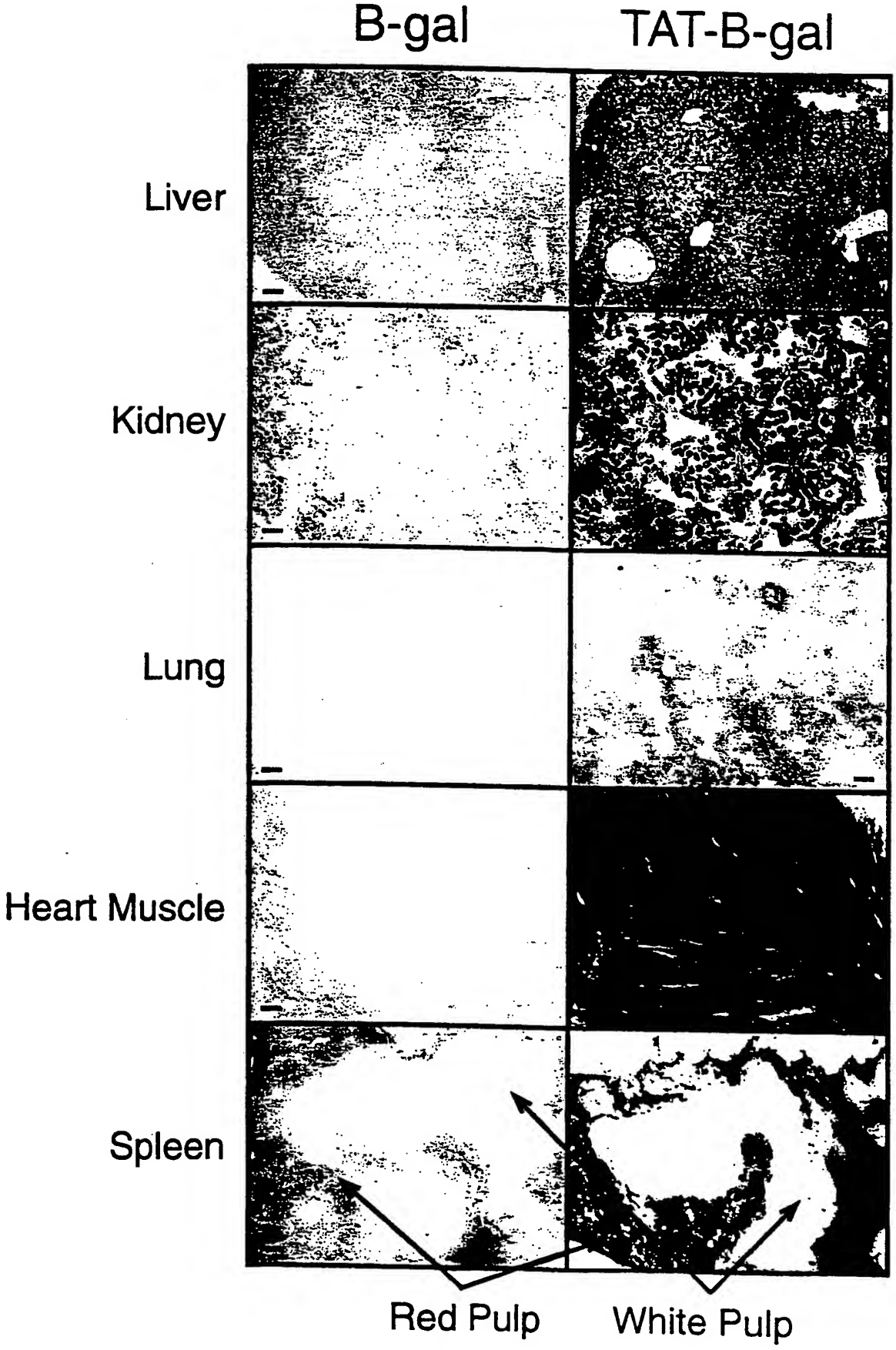


Fig 22A

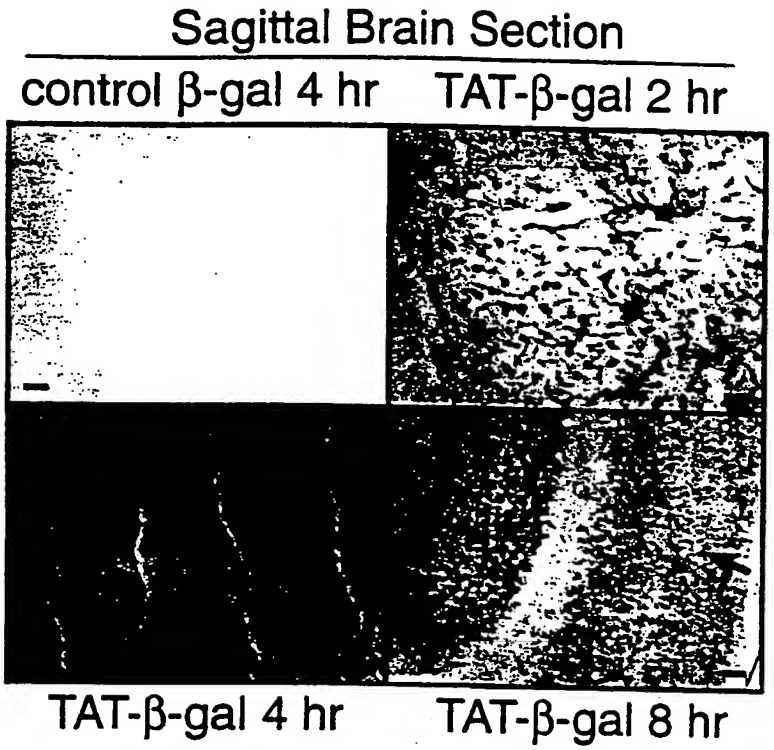
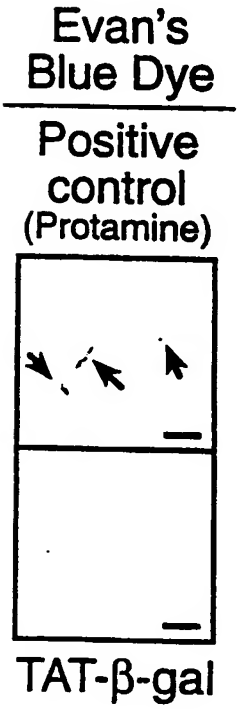


Fig 22B



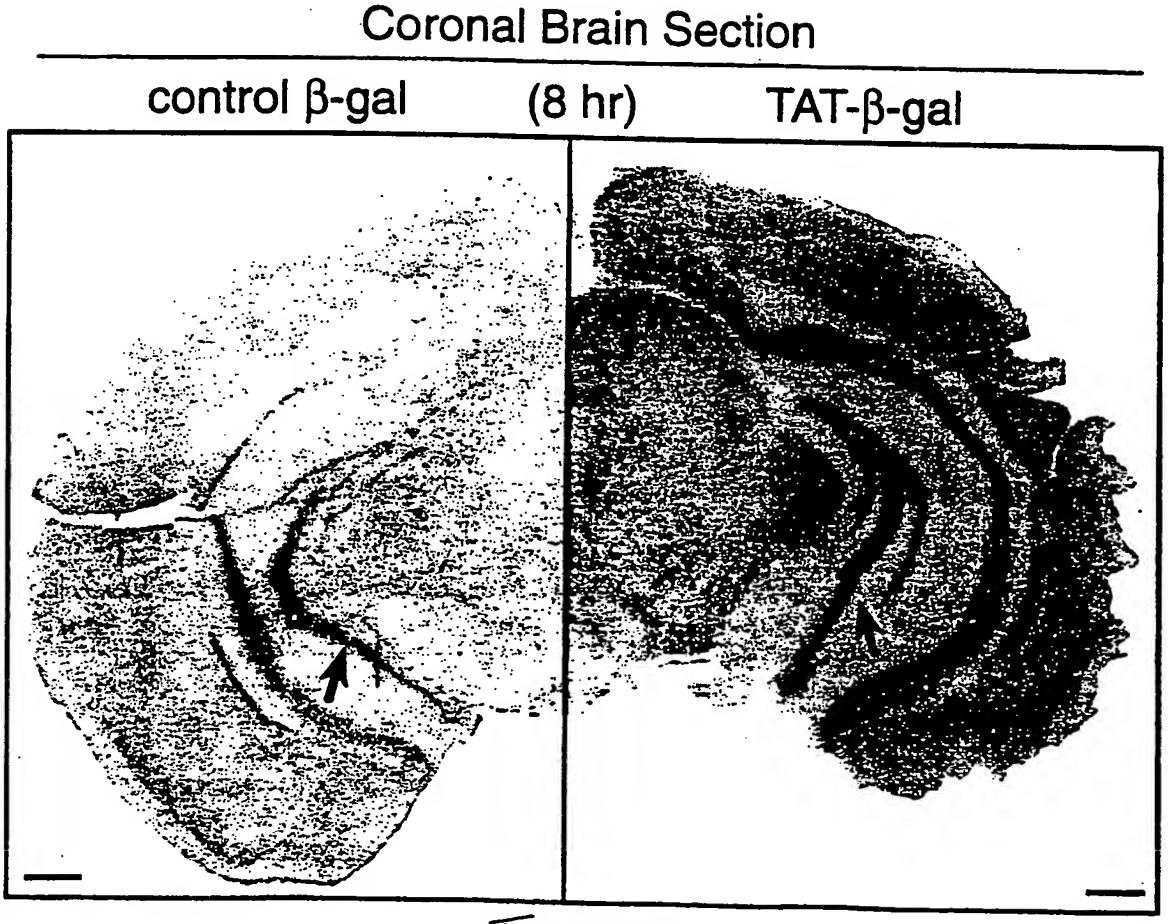


Fig 22C

Fig. 23C

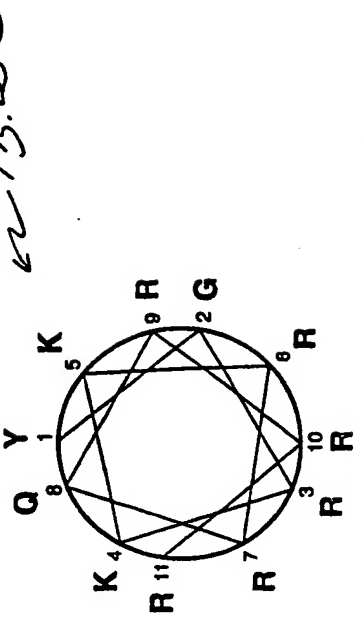


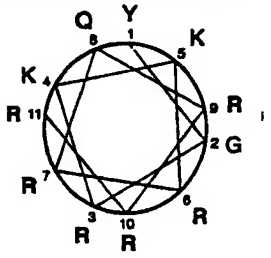
Fig. 23A

HIV TAT
(aa #47-57)
YGRKKRRQRRR

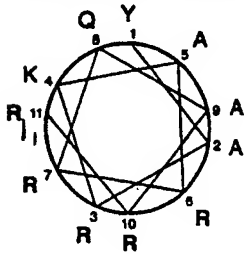
Fig. 23B

Fig. 24

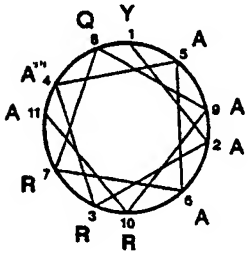
TAT (1x)
YGRKKRRQRRR



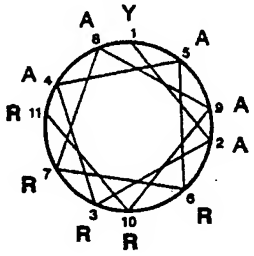
PTD-3 (5x)
YARKARRQARR



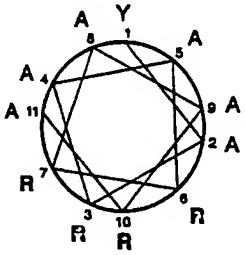
PTD-4 (33x)
YARAAARQARA



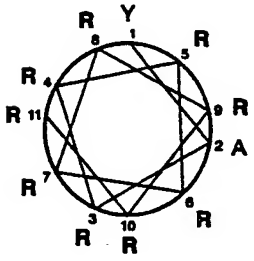
PTD-5 (8x)
YARAARRAARR



PTD-6 (5x)
YARAARRAARA



PTD-7 (1x)
YARRRRRRRRR



PTD-8 (<1x)
YAAARRRRRRR

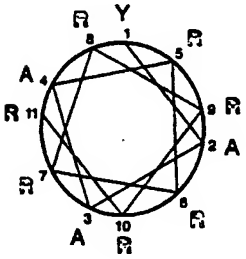


Fig. 25A

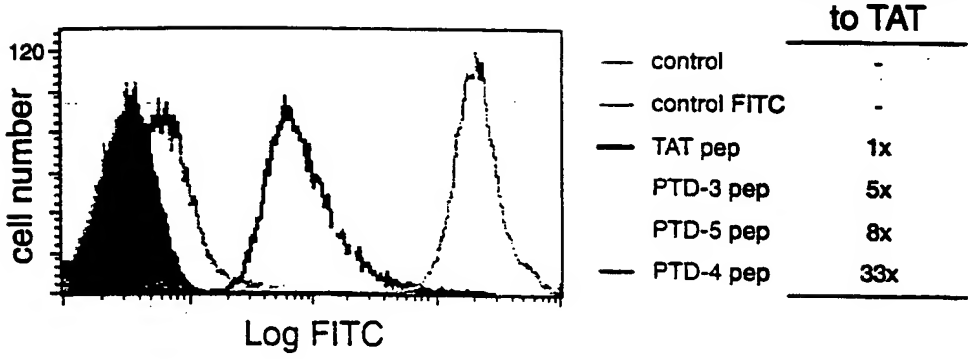
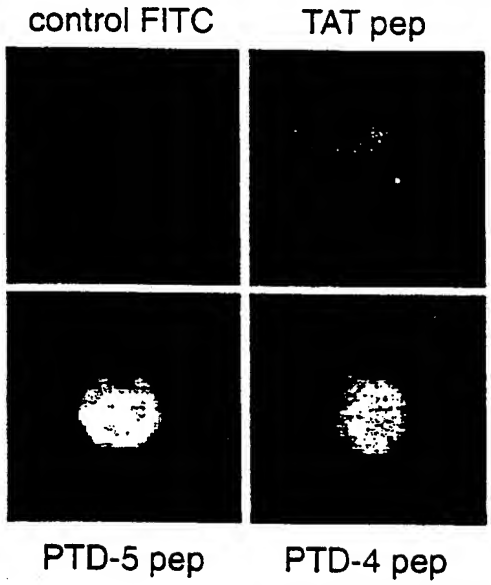


Fig. 25B



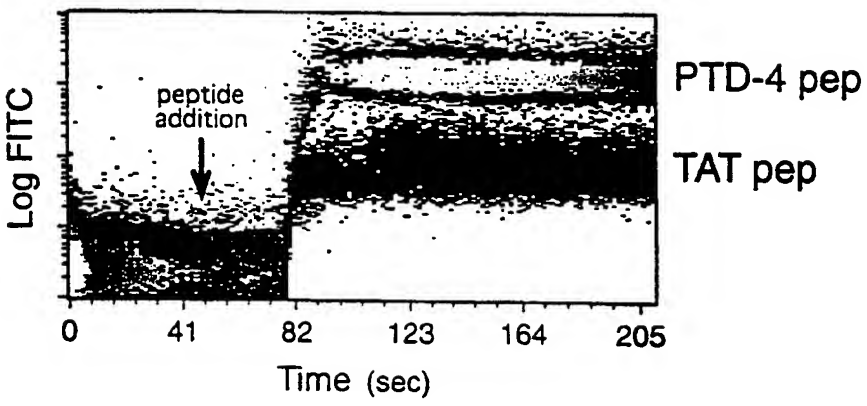
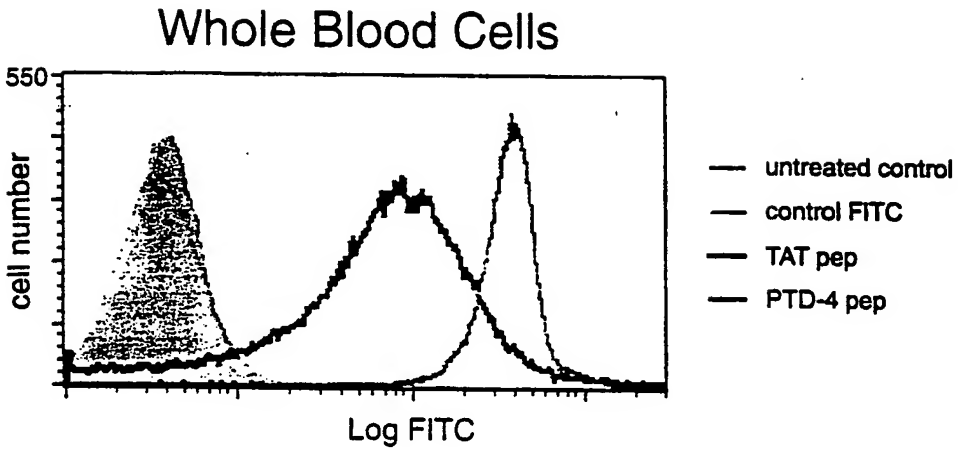


Fig. 25C

Fig. 26A



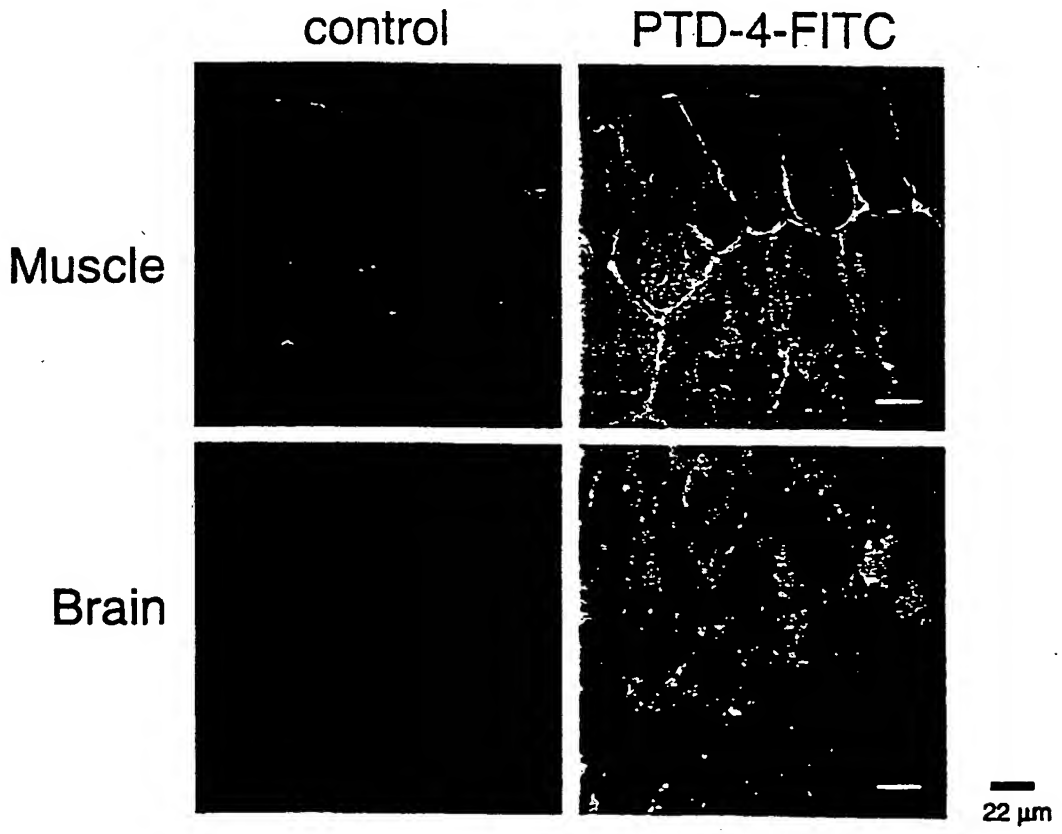
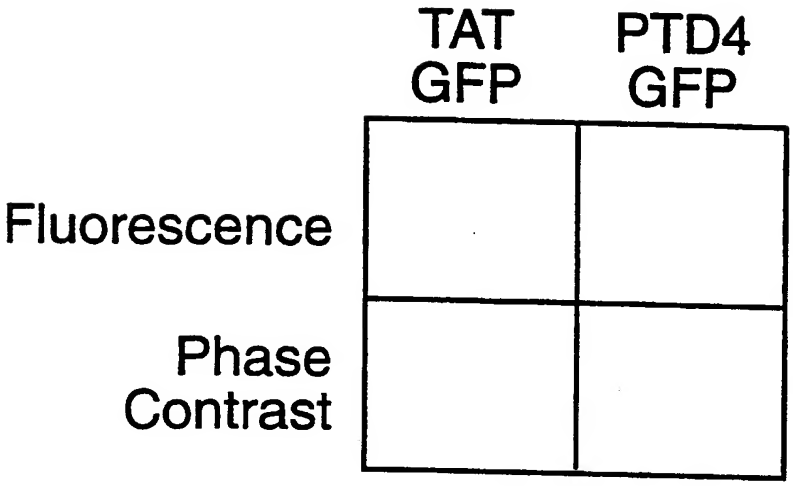


Fig. 26B

Fig. 27A



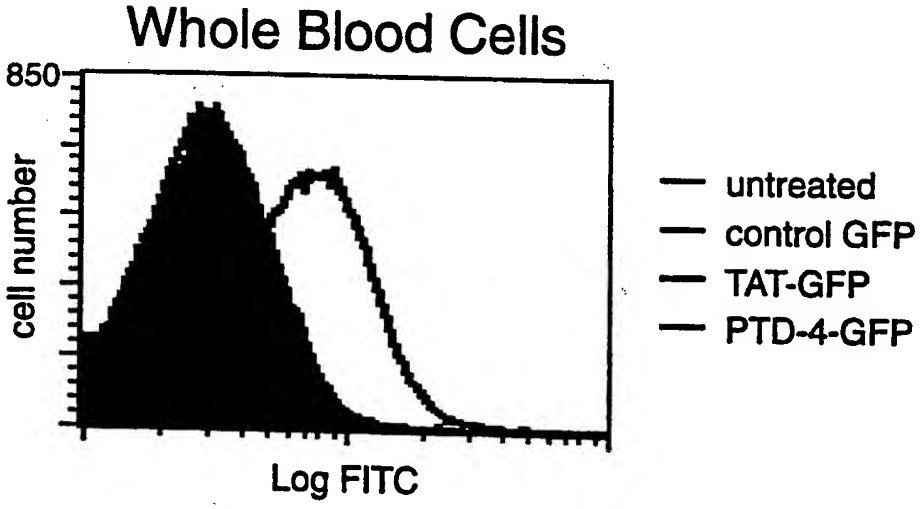


Fig. 27B

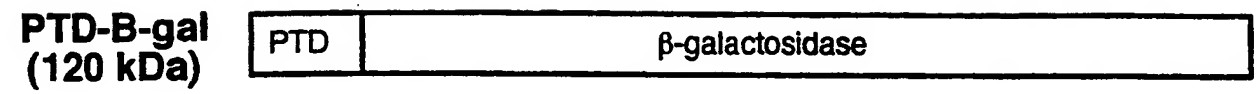


Fig. 28A

Rapid Transduction, but Slow Refolding of bacterial PTD-B-Gal (120 kDa)

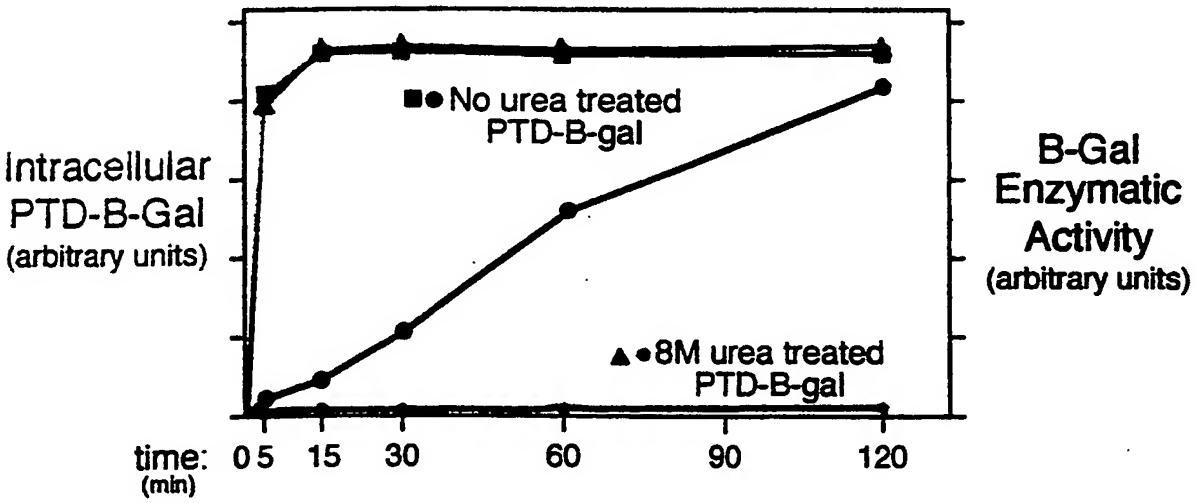


Fig. 28B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/05097

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/567

US CL : 435/7.21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: medline biosis caplus embase biotechds

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EZHEVSKY et al. Hypo-phosphorylation of the retinoblastoma protein (pRb) by cylin D:Cdk4/6 complexes results in active pRb. Proc. Nat. Acad. Sci. USA. 30 September, 1997, Vol 94, No. 20, pages 10699-10704, see especially abstract.	1, 2, 41
X -- Y -- A	Database Medline on STN, US National Library of Medicine (Bethesda, MD, USA) No. 1999098305, VOCERO-AKBANI et al. 'Killing HIV-infected cells by transduction with an HIV protease-activated caspase-3 protein'. Nature Medicine. January, 1999, Vol. 5, No. 1, pages 29-33, abstract only.	1, 2, 9, 41-43, 108-110 --- 45-47 --- 3-8, 10, 13, 44, 48-107

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not ir conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 AUGUST 2000	Date of mailing of the international search report 22 SEP 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RICHARD SCHNIZER Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 98/51325 A2 (CYTOGEN CORPORATION) 19 November, 1998, see entire document, especially abstract; page 40, lines 21-36; and page 41, lines 3,4, and 26-29.	1, 2, 5, 13, 41-43 ---------- 45
X -- Y	DONNELLY et al. Targeted delivery of peptide epitopes to class I major histocompatibility molecules by a modified Pseudomonas exotoxin. Proc. Nat. Acad. Sci. USA. April 1993, Vol. 90, pages 3530-3534. see especially abstract, and page 3533, third full paragraph.	1, 2, 5, 6, 9, 41 ---------- 7,8
Y -- A	ELLIOTT et al. Intercellular Trafficking and Protein Delivery by a Herpesvirus Structural Protein. Cell. 24 January 1997, Vol. 88, pages 223-233, see entire document, especially abstract.	1-10, 13, 10-47, 108-110 ---------- 48-55, 106, 107, 114-116
X	US 5,672,683 A (FRIDEN et al) 30 September 1997, see entire document, especially abstract; claims 1-11 at columns 42 and 43; and column 4, lines 19-28.	1-4
Y -- A	US 5,328,984 A (PASTAN et al) 12 July 1994, see entire document, especially abstract; claims 2-13 at columns 19 and 20, and claim 1 on the certificate of correction.	1-10, 13, 41, 45-47, 108-112 ---------- 48-55, 106, 107, 113-116
Y	FAWELL et al. Tat-mediated delivery of heterologous proteins into cells. Proc. Nat. Acad. Sci. USA. January 1994, Vol. 91, pages 664-668, see entire document, especially abstract.	1-10, 13, 41, 45-47, 108-110

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/05097

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 11, 12, 14-40, 42-44, 56-105, 111-112
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s)1-10, 13, 41-44, and 108-110, drawn to a fusion molecule and a first method of use.
Group II, claim(s) 45-55, drawn to a transgenic animal comprising a fusion molecule.
Group III, claim(s)113-116, drawn to a second method of using a fusion molecule.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The linking technical feature of groups I-III is a fusion molecule comprising a protein transduction domain linked to another molecule which is suspected of having therapeutic value. Ezhevsky et al teach a protein transduction domain linked to p 16(INK4A). See Proc. Nat. Acad. Sci. 94(20): 10699-10704, 9/1997. Fang et al teach that p16(INK4A) may be useful in gene therapy. See Oncogene 16(1): 1-8, 1-1998. Thus Ezhevsky teaches a composition comprising a protein transduction domain linked to a molecule which is suspected of having therapeutic valus. So, the linking technical feature of groups I-VIII cannot be a special technical feature under PCT Rule 13.2 because it does not constitute a contribution over the prior art.

The special technical feature of group I is considered to be a fusion molecule comprising at least one protein transduction domain and a linked molecule which is known or suspected to have the capacity to treat or prevent a medical condition in a mammal, and a method of using the fusion molecule treat or prevent a medical condition.

The special technical feature of group II is considered to be a transgenic mammal comprising a fusion molecule.

The special technical feature of group III is considered to be a second method of the fusion molecule, specifically for killing or damaging microbes.